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Specification and Drawings, as originally filed with Application for Patent Serial No: **2,220,805**, on November 12, 1997, by **MCGILL UNIVERSITY**, assignee of Moshe Szyf, for "DNA Demethylase, Therapeutic and Diagnostic Uses Thereof".

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ABSTRACT OF THE INVENTION

The present invention relates to a DNA demethylase enzyme having about 200 to about 240 Kbp, and wherein said DNA demethylase enzyme is overexpressed in cancer cells and not in normal cells.

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DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel enzyme, DNA demethylase, therapeutic and diagnostic uses thereof.

(b) Description of Prior Art

Many lines of evidence have established that modification of cytosine moieties residing in the dinucleotide sequence CpG in vertebrate genomes is involved in regulating a number of genome functions such as parental imprinting, X-inactivation, suppression of methylation of ectopic genes and differential gene expression (Szyf, 1996). DNA methylation performs its function of differentially marking genes because the distribution of methylated CpGs is tissue- and site- specific forming a pattern of methylation (Razin and Szyf, 1984). It is clear that the pattern of methylation is fashioned by a sequence of methylation and demethylation events (Brandeis et al., 1993) during development and is maintained in the fully differentiated cell (Razin and Riggs, 1980; Stein et al., 1982). While it was originally suggested that DNA demethylation is accomplished by a passive loss of methyl groups during replication (Razin and Riggs, 1980), it is now clear that an active process of demethylation occurs in embryonal cells (Frank et al., 1991), in differentiating cell lines (Razin et al., 1986; Szyf et al., 1985) and in response to estrogen treatment (Saluz et al., 1986). Two modes of demethylation have been documented: site specific demethylation that coincides in many instances with onset of gene expression of specific genes (Benvenisty et al., 1985) and a general genome wide demethylation that occurs during early development *in vivo* during

cellular differentiation and in cancer cells (Feinberg and Vogelstein, 1983; Razin et al., 1986; Szyf et al., 1985). The global demethylation is consistent with the hypothesis that a general demethylase activity which is activated at specific points in development or oncogenesis exists. It has been hypothesized that one mechanism regulating the pattern of methylation is the control of expression of methyltransferase (Szyf, 1991) and demethylase activities (Szyf et al., 1994). Although extensive information has been obtained on the enzymatic activity responsible for methylation and the regulation of its expression in the last two decades (Szyf, 1996), the identity of the demethylase has remained a mystery. It is clear however that to fully understand how patterns of methylation are formed and maintained and to determine their role in development, physiology and oncogenesis, one has to identify the demethylase enzyme(s). Two main difficulties have inhibited the identification of this enzyme. First, it is believed that demethylation of a methylated cytosine is chemically highly unlikely since it involves breaking a very stable C-C bond. Second, demethylation occurs at very defined stages in development (Brandeis et al., 1993) and identifying an adequate tissue source for this enzyme is critical.

Whereas no bona fide demethylase has been identified to date, alternative biochemical mechanisms involving exchange of methylated cytosines with nonmethylated cytosines have been described. One previously proposed mechanism is removal of the methylated base by a glycosylase and its replacement with a nonmethylated nucleotide utilizing an "excision-repair" mechanism (Razin et al., 1986). Glycosylase activities that can remove methylated cytosines from DNA have been demonstrated by Vairapandi and Duker

(Vairapandi and Duker, 1993) and more recently by Jost (Jost et al., 1995). However it is not clear whether this activity is responsible for the general demethylation observed in cellular differentiation. The fact that the activity identified by Jost acts specifically on hemimethylated sequences (which is not the natural substrate in most cases) and can remove thymidines as well as 5-methylcytosines, supports a repair function for this glycosylase-demethylase (Jost et al., 1995). An alternative mechanism involving a RNA dependent activity has been recently described by Weiss et al. (Weiss et al., 1996). This proteinase-insensitive RNA dependent activity has been shown to catalyze the excision and replacement of a methylated CpG dinucleotide with a nonmethylated CpG dinucleotide that is contained in a DNA-RNA hybrid molecule (Weiss et al., 1996). This activity which was identified in differentiating cells in culture was proposed to be involved in demethylation during development. In spite of these findings, the question of whether a bona fide demethylase exists remains open. However, it is not evident that Ras expression in p19 cells does reflect the situation in cancer cells. P19 is an embryonic cell and expression of Ras might be differentiate then.

It has been previously proposed that the extensive hypomethylation observed in cancer cells might be a consequence of activation of demethylase activity by oncogenic pathways (Szyf, 1994; Szyf 1995). In accordance with this hypothesis we have shown that ectopic expression of v-Ha-ras had induced demethylation activity in the cells (Szyf et al., 1995). Using an assay that directly measures the conversion of 3'^{32}P labeled methyl dCMP (mdCMP) into dCMP, we have shown that nuclear extracts prepared from P19-Ras transfectants bear high levels of demethylase

activity (Szyf et al., 1995). Building on this observation, we hypothesized that cancer cell lines were a good source for demethylase.

It would be highly desirable to be provided with a bona fide DNA demethylase (DNA dMTase).

SUMMARY OF THE INVENTION

In accordance with the present invention, we demonstrate the purification of a bona fide DNA demethylase (DNA dMTase) from a human lung cancer cell line A549, determine its kinetic parameters and substrate specificity. The DNA dMTase activity identified in this study converts methyl-dCMP (mdCMP) residing in the dinucleotide sequence mdCpG into dCMP whereas the methyl group is released as a volatile residue. The activity is purified away from any trace amounts of dCTP, is insensitive to the DNA polymerase inhibitor ddCTP, is not affected by the presence of methyl dCTP (mdCTP) in the reaction and does not exhibit exonuclease or glycosylase activities. The identification of this new enzyme points out to new directions in our understanding of how DNA methylation patterns are formed and altered.

More precisely and in accordance with the present invention there is provided a DNA demethylase enzyme having about 200 to about 240 Kbp, and wherein the DNA demethylase enzyme is overexpressed in cancer cells and not in normal cells.

In accordance with the present invention there is provided the use of an antagonist or inhibitor of DNA demethylase for the manufacture of a medicament for cancer treatment, for restoring an aberrant methylation pattern in a patient DNA, or for changing a methylation pattern in a patient DNA.

Such an antagonist is a double stranded oligonucleotide that inhibits demethylase at a Ki of 50nM, such as $\begin{bmatrix} C^mGC^mGC^mGC^mG \\ G^mCG^mCG^mCG^mC \end{bmatrix}_n$.

The inhibitor include, without limitation an anti-DNA demethylase antibody or an antisense of DNA demethylase.

The change of the methylation pattern may activate a silent gene. Such an activation of a silent gene permits the correction of genetic defect such as found for β-thalassemia or sickle cell anemia.

The DNA demethylase of the present invention may be used to remove methyl groups on DNA in vitro.

The DNA demethylase of the present invention or its cDNA may be used, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

The DNA demethylase of the present invention or its cDNA may be used, for inhibiting methylation in cancer cells using vector mediated gene therapy.

In accordance with the present invention there is provided an assay for the diagnostic of cancer in a patient, which comprises determining the level of expression of DNA demethylase of the present invention in a sample from said patient, wherein overexpression of the DNA demethylase is indicative of cancer cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A to 1G illustrate the purification of demethylase (DNA dMTase) from human A549 cells, presence of DNA dMTase in human H446 cells and its absence in human skin fibroblast cells;

Figs. 2A to 2C illustrate that DNA dMTase is a protein inhibited by RNA and not by ddCTP, mdCTP and is not affected by redox factors;

Figs. 3A and 3B illustrate the kinetics of DNA dMTase activity;

Fig. 4 illustrates the determination of maximum initial velocity and concentration of methylated DNA;

Figs. 5A and 5B illustrate the DNA dMTase activity exhibits no exonuclease or glycosylase activity;

Fig. 6 illustrates the remethylation of demethylated DNA;

Figs. 7A and 7B illustrate the demethylation involves transfer of a hydrogen from water to regenerate cytosine; and

Figs. 8A and 8B illustrate the substrate Specificity of DNA dMTase.

DETAILED DESCRIPTION OF THE INVENTION

The pattern of methylation is fashioned during development by a sequence of methylation and demethylation events. The identity of the demethylase has remained a mystery and alternative biochemical activities have been shown to demethylate DNA. Utilizing human lung carcinoma cells as a source for demethylase activity we demonstrate that mammalian cells bear a bona fide DNA demethylase (DNA dMTase) activity. DNA dMTase transforms methyl-C to C by catalyzing replacement of the methyl group on the 5 position of C with a hydrogen derived from water. DNA dMTase activity is chromatographically purified to one polypeptide without loss of activity and it does not copurify with DNA MeTase. DNA dMTase demethylates both fully methylated and hemimethylated DNA, shows dinucleotide specificity and can demethylate mdCpdG sites in different sequence contexts. This enzyme is different from previously described demethylation

activities: it is proteinase sensitive, activated by RNase and releases different products.

DNA dMTase is a novel enzyme showing a new and unexpected activity that has not been previously described in any organism. The finding of a bona fide demethylase, points out new directions in our understanding of the biological role of DNA methylation.

In spite of the fact that it was previously shown that Ras expression in p19 cells can induce demethylation activity, it was surprising to see that demethylation activity is presentin cancer cells. The finding of high levels of demethylase in A259 cells is indeed an unexpected discovery.

EXPERIMENTAL PROCEDURES

Cell Culture

A 549 Lung Carcinoma cells (ATCC: CCL 185) were grown in Dulbecco's modified Eagle's medium (with low glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 U/ml cifrofloxacin. Human Skin Fibroblasts #72-213A MRHF were obtained from BioWhittaker, Bethesda and were grown in Dulbecco's modified Eagle's medium supplement with 2% fetal calf serum, 2 mM glutamine. H446 Lung carcinoma cells (ATCC: HTB 171) was grown in RPMI 1640 medium with 5% fetal calf serum.

Preparation of nuclear extract

Nuclear extracts were prepared from A549 cultures at near confluence as previously described (Szyf et al., 1991; Szyf et al.,1995). The cells were trypsinized, collected and washed with phosphate-buffered saline and suspended in buffer A (10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 5mM KCl, 0.5% NP-40) at the concentration of 108 cells per ml for 10 min. at 4ºC.

Nuclei were collected by centrifugation of the suspension at 1000 g for 10 minutes. The nuclear pellet was resuspended in buffer A (400 µl) and collected as described in the experimental procedures. A nuclear extract was prepared from the pelleted nuclei by suspending them in buffer B (20 mM Tris, pH 8.0, 25% glycerol, 0.2 mM EDTA and 0.4 mM NaCl) at the concentration of 3.3×10^8 nuclei per ml and incubating the suspension for 15 min. at 4ºC. The nuclear extract was separated from the nuclear pellet by centrifugation at 10,000g for 30 minutes. Nuclear extract were stored in -80ºC for at least two months without loss of activity.

Chromatography on DEAE-Sepharose

A freshly prepared nuclear extract (1 ml , 1.1 mg) was passed through a Microcon™ 100 spin column, the retainant was diluted to a conductivity equivalent to 0.2 M NaCl in buffer L and applied onto a DEAE-Sepharose column (Pharmacia) (1.0 x 5 cm) that was preequilibrated with buffer L (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 0.2 M NaCl at a flow rate of 1 ml/min. The column was then washed with 15 ml of the starting buffer (buffer L + 0.2 M NaCl) and proteins were eluted with 5 ml of a linear gradient of NaCl (0.2-5.0 M). 0.8 ml fractions were collected and assayed for demethylase activity after desalting through a Microcon™ 10 spin column (Amicon) and resuspension of the retainant in 0.8 ml buffer L. DNA demethylase eluted between 4.9-5.0 M NaCl.

Chromatography on S-Sepharose

Active DEAE-Sepharose column fractions were pooled, adjusted to 0.1 M NaCl by dilution and loaded onto an S-sepharose column (Pharmacia) (1.0 x5 cm) which had been preequilibrated with buffer L containing 0.2 M NaCl at a flow rate of 1 ml/min. Following

washing of the column as described in experimental procedures, the proteins were eluted with 5 ml of a linear NaCl gradient (0.2-5.0M). 0.5 ml fractions were collected and assayed for DNA demethylase activity after desalting and concentrating to 0.2 ml using a Microcon™ 10 spin column. DNA demethylase activity eluted around 5.0 M NaCl.

Chromatography on Q-Sepharose

Active fractions from S-sepharose column were pooled, adjusted to 0.2 M NaCl by dilution and applied onto a Q-sepharose (Pharmacia) column (1.0 x5 cm) which had been equilibrated as described in the experimental procedures at a flow rate of 1 ml/min. The column was washed and the proteins were eluted with a linear NaCl gradient (0.2- 5.0 M). Fractions (0.5 ml) were collected, assayed for demethylase activity after desalting and concentrating to a final volume of 0.2 ml as described in the experimental procedures. The demethylase activity eluted around 4.8-5.0 M NaCl.

Gel-Exclusion Chromatography on DEAE-Sephacel

The pooled fractions of Q-sepharose column were adjusted to 0.2 M NaCl, loaded onto a 2.0 x 2.0 cm DEAE-Sephacel column (Pharmacia) and eluted with 10 ml of buffer L containing 0.2 M NaCl. The fractions (0.8 ml) were collected and assayed after concentration to about 180 µl with a Microcon™ 10 spin column for DNA demethylase activity. The activity was detected at fraction 4, which is very near the void volume (~200kDa).

Assay of DNA demethylase activity

To directly assay DNA demethylase activity *in vitro* two independent methods were applied.

(A) To assay the conversion of methyl-dCMP (mdCMP) to dCMP we used a previously described method (Szyf et al., 1995). Briefly, $\alpha^{32}P$ labelled, fully methylated

poly[mdC^{32}PdG]n substrate was prepared as follows. One hundred ng of a double-stranded fully methylated (mdCpdG) oligomer (Pharmacia) were denatured by boiling, which was followed by partial annealing at room temperature. The complementary strand was extended with Klenow fragment (Boehringer Mannheim) using methyl-5-dCTP (mdCTP, 0.1 mM) (Boehringer Mannheim) and [α-^{32}P] GTP (100 µCi, 3000 Ci/mmol), and the unincorporated nucleotides were removed by chromatography through a NAP-5 column (Pharmacia). The NAP-5 chromatography was repeated to exclude minor contamination with unincorporated nucleotides. As a control a nonmethylated poly[dC^{32}pdG]n substrate was similarly prepared except that a nonmethylated dCpdG oligomer served as a template and dCTP was used in the extension reaction. The column fractions (30 µl), described in the experimental procedures were incubated with 1 ng of poly[mdC^{32}pdG]n substrate for 1 hour at 37ºC in a buffer L containing 25% glycerol (v/v) and 5 mM EDTA. The reacted DNA as well as a nonmethylated poly[dC^{32}pdG]n and methylated [mdC^{32}pdG]n nonreacted controls were purified by phenol/chloroform extraction and subjected to micrococcal nuclease digestion (100 µg at 10 µl) and calf spleen phosphodiesterase (2µg) (Boehringer) (Pharmacia) to 3' mononucleotides for 15 hours at 37ºC. The digestion products were loaded onto a thin layer chromatography plate (TLC) (Kodak, 13255 Cellulose), separated in a medium containing, 132 ml Isobutyric acid:40 ml water: 4 ml ammonia solution, autoradiographed and the intensity of the different spots was determined using a phosphorimager (Fuji, BAS 2000). ^{32}P labelled substrates and tritium labelled substrates were phosphoimaged using BAS 2000 plate and BAS-TR2040 phosphorimager plate respectively.

(B) The second method determined removal of methylated residues from methylated DNA by measuring disappearance of ^{3}H-CH_3 or ^{14}C-CH_3 from the reaction mixture. 100 ng of poly [dCdG]n double stranded DNA was methylated using SssI methylase (New England Biolabs) and an excess of [^{3}H-methyl AdoMet (80 Ci/mmol; New England Nuclear)]. The tritiated methyl group containing DNA was purified from labeled AdoMet using NAP-5 column chromatography. All column purified fractions of DNA demethylase were assayed using the tritiated substrate. In a typical assay, 1 ng of DNA was incubated (at a specific activity of 4 $x10^{6}$dpm/mg)with 30 µl of column fraction for one hour at 37ºC in buffer L. To determine the number of methyl groups remaining in the DNA following incubation with the different fractions, 250 µl of water were added and the mixture was incubated at 65ºC for 5 minutes. One hundred µl of the reaction mixture were withdrawn for liquid scintillation counting. Controls received similar treatment except that in place of a column fraction, an equal volume of buffer L was added. The number of methyl groups that were removed from the DNA by the different fractions was determined by subtracting the remaining counts in each of the fractions from the counts remaining in the control. All tests were carried out in triplicates. The results are presented as picomole methyl group removed. One unit of DNA dMTase activity is defined as: amount of enzyme that releases one picomole of methyl group from methylated dCpdG substrate in one hour at 37ºC.

Methyl removal assay using double-labelled substrates

To determine whether the methyl group leaves the DNA and not any non-specific removal of tritium, we prepared SK plasmid DNA containing a tritiated hydrogen at the 6' position of cytosine and thymidine

by growing the plasmid harboring bacteria in the presence of deoxy [6-^{3}H] Uridine (22 Ci/mmol; Amersham) (10μCi/ml). The [6-^{3}H]-cytosine containing pBluescript SK(+) was purified according to standard protocols and was methylated using an excess of [^{14}C-methyl] AdoMet (59 mCi/mmol; Amersham) (10 μCi per 100 μl reaction)and SssI methylase. The double labeled DNA substrate was purified twice on a NAP-5 column. 15 μl of DNA dMTase were incubated with 1 ng of double labeled DNA (specific activity of 2000 dpm/ng) for 1 hour at 37ºC. Following incubation, the remaining ^{14}C versus ^{3}H counts were determined as described in the experimental procedures by scintillation counting (Wallac). The ^{14}C counts were normalized against ^{3}H counts. The controls received similar treatment except that instead of DNA dMTase, an equal amount of distilled water was added to them.

To determine the number of ^{3}H-CH$_3$ in the gaseous phase, 1 ng of ^{3}H-CH$_3$ poly [dCpdG] DNA were incubated with DNA dMTase overnight in a sealed tube (Pierce, Illinois, USA). 0.8 ml of air were removed from the tube using a gas tight syringe (Hamilton, Reno, Nevada) and injected into a sealed gas tight scintillation vial containing 10 ml OptiPhase scintillation fluid (Wallac, UK) and counted. As a control the DNA was incubated with an equal volume of buffer L and treated similarly.

Synthesis of other methylated dC dinucleotides

Poly [mdC^{32}pdA] and [mdC^{32}pdT] substrates were prepared as follows. About 0.5 μg of 20 mer oligonucleotides 5'(GG)103', 5'(GT)103' and 5'(GA)103' were boiled and annealed at room temperature with oligonucleotide 5'CCCCCC3', 5'CACACA3' and 5'CTCTCT3' respectively. The complementary strand was extended with Klenow fragment using m5dCTP (Boehringer

Mannheim) and either [α^{32}P] dATP (100μCi, 3000Ci/mmol) or [α^{32}P] dTTP (100 μCi, 3000 Ci/mmol) respectively. The unincorporated nucleotides were removed by chromatography through a NAP-5 column. Hemimethylated mdCpG substrate was prepared in a similar manner except that a nonmethylated poly dCpdG substrate (Boehringer) was used as template and m5dCTP and [α^{32}P]dGTP were used for extension as described in the experimental procedures.

Assay for nuclease and glycosylase activity

[^{32}pmdCpdG]n substrate which included a labeled ^{32}P 5' to mdC was prepared as follows. About 100 ng of poly dCpdG DNA were boiled and partially annealed at room temperature. [α^{32}P]dCTP and cold dGTP were used for complementary strand extension as described in the experimental procedures. The free nucleotides were separated using NAP-5 column chromatography. The purified [^{32}pmdCpdG]n DNA was subjected to methylation by SssI methylase using 320 μM AdoMet. The DNA was repurified twice using a NAP-5 column. The methylated DNA (1 ng) was incubated with either 30 μl DNA dMTase, nuclear extract or buffer L. To determine whether α^{32}P labeled residue is excised from the DNA it was directly applied (3μl) onto a TLC plate. To determine whether the DNA was demethylated it was subjected to digestion with snake venom phosphodiesterase (0.2 mg in a 10μl reaction volume) (Boehringer Mannheim) which attacks the 3'-OH group releasing 5'-mononucleotides. The resulting mononucleotides were separated on TLC plates and autoradiographed.

To test whether dCTP copurifies with DNA dMTase, which may be involved in activities other than bona fide demethylation, 20 μM of dCTP with 1 μl of α^{32}P labeled dCTP (3000 Ci/mmole) was loaded onto the

column with nuclear extract. The ^{32}P counts were measured in the flow through, washes and in the different fractions. About 1.1 million counts were loaded onto the DEAE-Sepharose column and were all recovered up to fraction 8.

To determine whether DNA dMTase contains a DNA polymerase activity, DNA demethylase reactions were performed in presence of 500 µM of ddCTP (Pharmacia) or 500 µM of m5dCTP (Boehringer Mannheim) at initial rate conditions.

To determine whether DNA dMTase is sensitive to RNase or Proteinase K treatment, DNA dMTase was pretreated for 1 h at 56ºC with 200 µg/ml proteinase K (Sigma). A demethylation reaction was carried out with this pretreated fraction in the usual manner using both demethylation assays described in the experimental procedures. To test the effect of RNA digestion on the demethylation reaction, the fractions from different columns were treated with 100 µg/ml RNase A (Sigma).

Demethylation of pBluescript SK(+) Plasmid

About 4 µg plasmid pBluescript SK (Stratagene) was subjected to methylation using SssI methylase. The methylated plasmid (4 ng) was incubated with 30 µl of DNA dMTase Fraction 4 of DEAE-Sephacel column under standard conditions, extracted with phenol: chloroform and precipitated with ethanol. About 1 ng of the plasmid were subjected to digestion with 10 units each of either of the restriction endonucleases EcoRII (GIBCO-BRL), DpnI, HhaI or HpaII (New England Biolabs) before and after methylation as well as after DNA dMTase treatment in a reaction volume of 10 µl for 2 hour at 37ºC. Following restriction digestion the plasmids were extracted with phenol:chloroform, ethanol precipitated and resuspended in 10 µl. The plasmids were electrophoresed on a 0.8% (w/w) Agarose gel,

transferred onto a Hybond Nylon membrane and hybridized with pBluescript SK(+) plasmid which was ^{32}P labelled by random-priming (Boehringer Mannheim).

Effect of Redox Reagents (NAD, NADH, NADP, NADPH and $FeCl_3$) on demethylase activity

The reagents were prepared at 100 µM concentration and added at a final concentration of 10 µM to a standard methyl removal assay under initial rate conditions as described in the experimental procedures. The methyl removal activity in presence of each of the cofactors was compared to a control DNA dMTase reaction.

Determination of kinetic parameters

For determination of kinetic parameters, the demethylation reactions were performed using both assays (generation of dCMP and removal of methyl) as described in the experimental procedures except that varying DNA concentrations from 0.1 mM to 2.5 mM were used in a total volume of 50µl including 30 µl of DNA dMTase. Since it has been established by previous experiments that the reaction proceeds for at least 3 hours, the initial velocity of reaction was measured at one hour intervals. The velocity data was collected at each substrate DNA concentration range stated for both assays. The Km and Vmax values for DNA demethylase activity were determined from double reciprocal plots of velocity versus substrate concentration.

Human A549 cells bear a demethylase activity that could be purified away from dCTP and DNA MeTase

The use of an appropriate cellular source and a direct assay for demethylase activity are obviously critical. As we have previously shown that demethylase activity was induced in response to ectopic expression of the Ras oncogene (Szyf et al., 1995) we reasoned that cancer cells might bear high levels of

demethylase activity. Based on preliminary studies demonstrating the presence of high levels of demethylase activity in the human lung carcinoma cell line A549, we have chosen this cell line for our further studies and purification steps. Previous studies have used indirect measures such as increased sensitivity to methylation-sensitive restriction enzymes as indicators of demethylase activity (Weiss et al., 1996; Jost et al., 1995). To directly measure the conversion of 5-mdCMP in DNA to dCMP, we have utilized a completely methylated ^{32}P labeled [mdC^{32}pdG]n double stranded oligomer which we had previously described (Szyf et al., 1995). Following incubation with the different fractions, the DNA is purified and subjected to cleavage with micrococal nuclease to 3' mononucleotides. The 3' labeled mdCMP and dCMP are separated by thin layer chromatography (TLC) and the conversion of mdCMP to dCMP is directly determined. This assay provides a stringent test for bona fide demethylation and discriminates it from previously described 5mCpC replacement activities (Jost et al., 1995; Weiss et al., 1996). The glycosylase-demethylase activity described by Jost et al. (Jost et al., 1995) will require the presence of a ligase activity and an energy source for replacement of mdC with C to be detected by our assay, whereas the demethylase activity described by Weiss et al. will not be detected since it replaces the intact mdC^{32}pdG dinucleotide with a cold dCpdG without altering its state of methylation (Weiss et al., 1996).

Nuclear extracts were prepared from A549 cells, applied onto a DEAE-Sepharose column, eluted with a linear gradient from 0.2-5.0M NaCl and the fractions were assayed for demethylase (dMTase) activity as described in the experimental procedures. As shown in

Fig. 1(A) a clear peak of dMTase activity is eluted at the high salt fraction 10.

Conversion of methylated cytosine to cytosine: Nuclear extracts prepared from A549 cells (1.1 mg) were passed through an AMICON™ 100 spin column. The retainant (98.56 mg, 0.2 mg/ml) was loaded onto a DEAE-Sepharose column, the different chromatographic column fractions eluted by a linear NaCl gradient (0.2-5M) were desalted and (30 µl) incubated with 1 ng of [mdC^{32}pdG]n double stranded oligomer for 1 hour at 37ºC, digested to 3' mononucleotides and analyzed on TLC as described in the experimental procedures. Control methylated (ME) and nonmethylated (NM) [dC^{32}pdG]n substrates were digested to 3' mononucleotides and loaded on the TLC plate to indicate the expected position of dCMP and mdCMP. The active fraction is indicated by an arrow. This fraction was loaded on S-Sepharose followed by Q-Sepharose and DEAE-Sephacel fractionation.

The first chromatography step purified the dMTase activity from the bulk of nuclear protein (Fig. 1B) and is a very effective purification step.

DNA dMTase activity as measured by the release of volatile methyl residues. The different column fractions were incubated with 1ng (4 x 10^6 dpm/µg) of [^{3}H]-CH_3-[mdCpdG]n oligomer and the release of volatile methyl residues was determined (-) and presented as units per mg protein (U/mg). The results are an average of three independent determinations. One unit equals release of one picomole of CH3 per hour at 37°C. DNA MeTase activity was determined using a hemimethylated DNA substrate and [3H]-AdoMet as previously described (-). One units equals the amount of enzyme that catalyses transfer of one picomole of tritiated methyl group on hemimethylated DNA per

minute. The result of an average of three independent determinations (+/- S.E). Protein concentration was determined using the Bio-Rad Bradford kit (-). The elution profile of 20 μM of [^{32}P]-α-dCTP incubated with the protein was determined by scintillation counting of the different DEAE fractions (-) and presented as fraction of dCTP loaded on the column.

To exclude the possibility that the DNA dMTase activity detected in our assay is carried by the DNA MeTase, we assayed the fractions for DNA MeTase activity using a hemimethylated DNA substrate as previously described (Szyf et al., 1991). As observed in Figure 1B DNA MeTase activity is detected in the second and third fractions, thus our fractionation separated DNA dMTase away from the DNA MeTase suggesting that they are independent proteins.

There is a remote possibility that the demethylation observed is not a bona fide demethylation but a consequence of a glycosylase removal of mC, followed by removal of the remaining deoxyribose-phosphate by AP (apyrimidine) nuclease, repair of the gap catalyzed by DNA polymeraseβ using trace dCTP contained in the fraction and ligation of the break with ligase in the presence of residual ATP. For this hypothesis to be consistent with our data, four independent enzymes and two cofactors have to cofractionate with DNA dMTase. To exclude the possibility that a trace amount of dCTP is bound to DNA dMTase active fraction, we have added 20 μM of ^{32}P labeled dCTP ($10x10^6$ cpm) to the nuclear extract and determined its elution profile on the DEAE column. Less than background cpm (10 cpm) were detected in the DNA dMTase active fraction suggesting that our first column purifies dCTP away from the DNA dMTase at least $1x10^6$ fold (Fig. 1B). If any dCTP is present in the

nuclear extract, the remaining concentration after fractionation on DEAE is well below the Kms of the known DNA polymerases (Dong et al., 1993). The possibility that dCTP is so tightly bound to the enzyme that it could not be replaced by the exogenous ^{32}P labeled dCTP is very remote since an enzyme using dCTP as substrate must readily exchange dCTP.

The active fraction 10 was further fractionated sequentially on the following columns: S-Sepharose and Q-Sepharose. The DNA dMTase eluted at the high salt fraction from both columns as determined by the [mdC^{32}pdG]n demethylation assay (Fig. 1A). The ion exchange chromatography was followed by gel-exclusion chromatography on DEAE-Sephacel. The activity was detected at fraction 4 which is near the void volume. Calibration of the column with MW size markers suggested that the demethylase is of the size of >200 kDa. The purification steps were verified by a silver-stained 10% PAGE analysis of the different fractions obtained following the DEAE-Sepharose column and the active fractions at each of the purification steps (Fig. 1C).

PAGE analysis of DEAE fractionation steps (20 µl) (left panel) analysed on 10% polyacrylamide gel (19:1), electrophoresed for 16 hours at 25 volts, fixed and stained with silver staining using Bio-Rad silver plus kit (left panel). The central panel shows a PAGE analysis of the active fractions (putative DNA dMTase is indicated with an arrow). The DEAE and DEAE-Sephacel active fractions were concentrated and further analyzed on PAGE (6.5 %). The position of DNA dMTase is indicated by an arrow. The relative migration of MW standards (Rainbow-Amersham) is indicated.

These results demonstrate a very effective purification of DNA dMTase away from most of the

nuclear protein at the DEAE fractionation step and apparent purification following the size exclusion step. The active DEAE-Sephacel fraction 4 was concentrated and analyzed on PAGE demonstrating a single main polypeptide migrating >200 kDa following silver staining. The fact that we have maintained our activity even after 4 fractionation steps and that only a single polypeptide is apparent after the last purification step argues strongly against the possibility that the activity detected in our study is a repair or replacement activity. Any replacement mechanism must involve a number of proteins and additional cofactors and substrates. In summary, the chromatography of the demethylase activity in A459 cells provides strong support to the hypothesis that mammalian cells bear a bona fide demethylase activity.

DNA dMTase releases a volatile derivative

A bona fide demethylation of 5-methylcytosine has to result in release of the methyl group as a volatile derivative. Previously described enzymatic demethylation reactions involve oxidation of the methyl group and result in release of CO_2, methanol (Bechtold et al., 1972) or methane (Sauer et al., 1977). An additional proposed derivative of demethylation reactions is formaldehyde (Huszti and Tyihak, 1986). To test the hypothesis that the demethylase activity characterized above results in the release of a volatile derivative, we have generated a [mdCpdG] oligomer bearing a ^{3}H labeled CH_3 group as described in the methods. The substrate (1ng) was incubated with the different column fractions and the rate of release of the tritiated methyl from the aqueous phase was determined by scintillation counting of the remaining radioactivity in the reaction mix. As demonstrated in Fig. 1B (the line indicated by a diamond) the same gel

chromatography fractions that demethylate the [mdC^{32}pdG]n substrate also release labeled methyl groups from the ^{3}H labeled [mdCpdG] oligomer as predicted. These results further support the conclusion that the DNA dMTase activity identified in our chromatography steps is a bona fide DNA dMTase.

To further determine that demethylase reaction involves release of the methyl per se and that the cytosine base ring remains in the aqueous phase, we synthesized a methylated pBluescript SK(+) plasmid substrate which had been doubly labeled with a ^{3}H-hydrogen at the sixth position of cytosine and with a ^{14}C at the CH_3 group as described in the experimental procedures. The substrate was incubated for different time points with the purified DNA dMTase and the quantity of residual ^{14}C and ^{3}H in the aqueous phase was determined by a liquid scintillation counter followed by a spectrum analysis to separate the ^{14}C and the ^{3}H counts. The results shown in Fig. 1D demonstrate that while the ^{14}C counts are released from the aqueous phase in a time dependent manner (no loss of counts was observed in control reactions that did not contain DNA dMTase) the ^{3}H counts remain in the aqueous phase. These results are consistent with the hypothesis that the CH_3 group is selectively released as a volatile residue whereas the cytosine remains intact.

DNA dMTase releases methyl residues from 5-position of cytosine (labelled with ^{14}C) but not the hydrogen at the 6-position (labelled with [^{3}H]) as determined using a double labelled pBluescript SK(+)-plasmid synthesized as described in the experimental procedures. One ng of the double labelled plasmid was incubated for different time points with the purified DNA dMTase (fraction 4 of DEAE-Sephacel) and the

quantity of residual ^{14}C and ^{3}H in the aqueous phase was determined by a liquid scintillation counter followed by a spectrum analysis to separate the ^{14}C and the ^{3}H counts. The results are presented as the ratio between the ^{14}C and ^{3}H counts. The results are an average of three independent determinations (+/- S.E).

We then determined whether the volatile residue could be detected in the gaseous phase. Our ^{3}H labeled [mdCpdG] oligomer substrate was incubated with DNA dMTase as described in the experimental procedures in a sealed tube. The gaseous phase was removed with a gas tight syringe and injected into a gas sealed scintillation vial. As observed in Fig. 1E an appreciable amount of ^{3}H counts are detected in the gaseous phase in comparison with a control reaction that does not include the DNA dMTase.

The presence of $[^{3}H]$-CH_3-residues in the gaseous phase following incubation with DNA dMTase. One nanogram of a $[^{3}H]$-CH_3-[mdCpdG]n oligomer was incubated with 30 µl of DNA dMTase (fraction 4) for 24 hours. As a control, the substrate was incubated in the absence of DNA dMTase in a sealed tube. Eight hundred µl of air was suctioned using a gas tight syringe and injected into a gas sealed scintillation vial containing scintillation fluid and counted in the scintillation counter. Results are presented as an average of three independent determinations +/- S.E..

Not all expected counts are recovered in the gaseous phase, possibly reflecting some loss of the gaseous phase during the transfer to the scintillation vial or reduced solubility of the volatile residue in the scintillation cocktail.

The velocity of removal of volatile methyl residue is dependent on substrate concentration and approaches saturation at higher concentrations of DNA

consistent with first order kinetics with respect to methylated DNA (Fig. 1F).

The initial velocity of removal of methyl groups from DNA is dependent on the concentration of methylated DNA. A DNA dMTase reaction was carried out for 1 hour at 37ºC in the presence of 30µl of purified DNA dMTase and a range of concentrations of [^{3}H]-CH$_3$-[mdCpdG]n oligomer as indicated. The rate of removal of volatile methyl residue from the DNA was determined by scintillation counting.

In summary, our results indicate that DNA dMTase releases the methyl group from m5dC in DNA as a volatile residue.

DNA dMTase activity is absent in nontransformed cells

To test the hypothesis that cancer cells express induced levels of DNA dMTase in comparison with nontransformed cells as we have previously proposed (Szyf et al., 1995), we compared the DNA dMTase activity in a DEAE fractionated nuclear extract prepared from a different human lung carcinoma cell line H446 to nontransformed human skin fibroblast cells. Equal concentrations of DEAE fraction 10 protein were assayed for DNA dMTase activity at initial rate conditions using [mdC^{32}pdG]n double stranded oligomer as a substrate. As observed in Fig. 1G, whereas DNA dMTase activity is readily observed in the two lung carcinoma cell lines, it is undetectable in nontransformed human cells.

DNA dMTase activity is absent in nontransformed human skin fibroblast cells. Nuclear extracts were prepared from human lung carcinoma cells H446 as well as from human skin fibroblast cells. Equal amounts of nuclear extracts (100 µg) were loaded onto a DEAE column and proteins were eluted with an NaCl gradient as described in the experimental procedures. Equal

concentrations of fraction 10 protein were assayed for DNA dMTase activity at initial rate conditions using [mdC^{32}pdG]n double stranded oligomer as a substrate. DNA dMTase prepared from A549 cells served as a positive control. A duplicate experiment is presented per extract. A longer exposure of the MHRF is shown to exclude any residual activity (indicated by a * in Fig. 1G).

This is consistent with the previously observed induction in DNA dMTase activity by oncogenic Ras in P19 cells (Szyf et al., 1995).

DNA dMTase is a protein which is inhibited by RNA and does not involve an exchange activity

It has recently been shown that a rat myoblast cell line bears a demethylase activity that is proteinase insensitive but is inhibited by RNase (Weiss et al., 1996). To exclude the possibility that the DNA dMTase activity that we have purified is identical to the previously described activity and to determine whether DNA dMTase is a protein, we subjected the DNA dMTase to either RNase or proteinaseK treatment. As observed in Fig. 2 the DNA dMTase activity measured either as transformation of mdC to C (Fig. 2A) or as release of volatile methyl residues (Fig. 2C) is abolished after proteinase K treatment and is not inhibited but rather enhanced after RNase treatment.

1 ng of [mdC^{32}pdG]n double stranded oligomer was incubated for 1 hour at 37°C with either buffer L (ME), active DNA dMTase fractions from the different chromatography steps in the presence of 100µg/ml RNase as indicated, with purified DNA dMTase in the presence of RNase and ddCTP (500 µM), with purified DNA dMTase in the presence of RNase and either mdCTP (500µM) or ddCTP (500 µM), with purified DNA dMTase in the absence of RNase, or with DNA dMTase following pre incubation with Proteinase K (200 µg/ml) (Fig. 2A). The reaction

products were digested to 3' mononucleotides and analyzed by TLC as described in the experimental procedures. Control methylated (ME) and nonmethylated (NM) [dC32pdG]n substrates were digested to 3' mononucleotides and loaded on the TLC plate to indicate the expected position of dCMP and mdCMP.

Demethylase activity is affected by RNase and Proteinase K (Fig. 2C). The release of volatile methyl residues was determined in presence and absence of RNase and Proteinase K and presented as percentage relative to the values obtained in the absence of DNA dMTase (control).

Thus the DNA dMTase identified here is a protein and not an RNA and is unequivocally different from the previously published (Jost et al., 1995; Weiss et al., 1996) demethylase activity.

To further exclude the possibility that DNA dMTase bears a m5dC: dC replacement activity, we tested the ability of 500 μM of ddCTP to inhibit demethylation of the [mdC^{32}pdG]n substrate. ddCTP will inhibit at this concentration DNA polymerase β (Fisher et al., 1979) which has been suggested by Jost et al. to be involved in the repair component of the glycosylase-demethylase activity (Jost et al., 1995). Moreover, since our assay only detects the 5' neighbors of [^{32}P]-dGMP, a replacing incoming C is detected only if it is ligated to a [^{32}P]-dGMP in the substrate. ddCMP can not form a phosphodiester bond with its 3' neighbor. As observed in Fig. 2A, dCMP does not inhibit DNA dMTase activity.

The remote possibility exists that a novel DNA polymerase is involved in replacement of the m5dC in our substrate with some residual C contained in the purified DNA dMTase which is not inhibited by or does not recognize ddCTP as a substrate. To exclude this

possibility, we have added a very significant excess of mdCTP (500 μM) to our demethylation reaction. Electroporation of vertebrate cells with mdCTP results in increased methylation of DNA (Holliday and Ho, 1991). If a replacement mechanism is involved in demethylation, the presence of mdCTP should result in incorporation of methylated cytosines and essential inhibition of demethylation. However, the results shown in Fig. 2A are inconsistent with this hypothesis as mdCTP does not inhibit demethylation.

In summary, the results of the experiments described in this section demonstrate that the DNA dMTase identified in this study is distinct from the demethylation activities identified by Jost et al., (Jost et al., 1995) and Weiss et al., (Weiss et al., 1996).

DNA dMTase activity is not dependent on redox factors and is not inhibited by non-specific RNA

Demethylation of steroids generates CO_2 and formate (Bechtold et al., 1972), this reaction requires NADPH as a cofactor. Does our DNA dMTase activity require NADPH or NADH? Steroid demethylation requires exogenously added NADPH while our reaction proceeds in the absence of exogenous NADPH or NADH. It is possible however that in spite of our extensive purification some NADPH/NADH is bound to the active site of the enzyme which drives the reaction. Since NADPH or NADH must be replenished to enable the reaction to proceed, it should be depleted rapidly unless exogenous NADPH/NADH is added. Supplementing the reaction with exogenous NADPH or NADH should enhance the initial rate of the reaction if it is dependent on these cofactors. As observed in Fig. 2B, none of the added redox factors alter the initial rate of DNA dMTase supporting the hypothesis that the enzyme does not require additional redox factors.

Demethylase activity is independent of Redox factors and not affected by non-specific RNA. Purified DNA dMTase (30 µl) was incubated with (1.5 ng 4 x 10^6 dpm/µg) of [^{3}H]-CH_3-[mdCpdG]n oligomer in buffer L for 1 h. at 37ºC (control) and in the presence of either 100µM of β-NAD, β-NADH, β-NADP, β-NADPH, $FeCl_3$, 100 µg/ml of yeast t-RNA and total RNA prepared from human A549 cells. The release of volatile methyl residues was determined and presented as percentage relative to the values obtained in the absence of these factors (a difference of 3750 dpm). The results of an average of three independent determinations (+/- S.E) are shown.

This is consistent with the fact that the reaction exhibits a constant velocity for an extended span of time (Figs. 3A and 3B).

1 ng of [mdC^{32}pdG]n double stranded oligomer was incubated for two hours with either buffer L (ME), or purified DNA dMTase (5 ml) for different time points as indicated. The reaction products were digested to 3' mononucleotides and analyzed by TLC as described in the experimental procedures. Control nonmethylated (NM) [dC^{32}pdG]n substrate was digested to 3' mononucleotides and loaded on the TLC plate to indicate the expected position of dCMP.

Kinetics of release of methyl residues and generation of dCMP by DNA dMTase. The amount of methyl residues released by DNA dMTase at different time points were determined using one nanogram of a [^{3}H]-CH_3-[mdCpdG]n oligomer as described above for Fig. 1. The results are a summary of three independent determinations +/-S.E as indicated (diamond). Chromatograms like those presented in Fig. 3A were quantified by a phosphorimager and the rate of transformation of mdCMP to dCMP was calculated. The

results are a summary of three independent determinations +/- S. E. (box).

To determine whether [^{14}C]-CO_2 is produced, we incubated the reaction mixture in a closed scintillation vial bearing an NaOH soaked filter. If CO_2 is produced, it should react with the NaOH on the filter to produce ^{14}C labeled $NaHCO_3$. No counts were detected on the filter. In summary, the mechanism of action of DNA demethylation is different from steroid demethylation, it does not require NADPH or other redox factors and consistent with this fact it does not generate CO_2. The methyl group might be oxidized and leave in the form of methanol (Toews et al., 1979) or formaldehyde (Huszti and Tyihak, 1986). Using dimedon as a formaldehyde trap in a previously described standard assay for formaldehyde (Huszti and Tyihak, 1986), we were not able to identify labeled ^{3}H formaldemethone generated in the demethylation of ^{3}H labeled [mdCpdG] DNA. In summary, demethylation does not require redox cofactors and does not generate some of the previously described oxidized residues.

As the demethylation reaction is activated by RNase, we determined whether the enzyme is nonspecifically inhibited by RNA. As shown in Fig. 2B yeast tRNA or total RNA from human cells does not inhibit DNA dMTase suggesting that the enzyme is inhibited by specific form of RNA.

Kinetic parameters of DNA dMTase

The DNA dMTase reaction proceeds without any requirement for additional substrates such as dCTP or redox factors. However, it is possible that a small number of molecules of these putative substrates are tightly bound to the enzyme itself. To further determine whether the enzyme is dependent on a nonreplenishable substrate that is tightly bound to the

enzyme such as dCTP or a redox factor such as NADH or NADPH, we determined the kinetic parameters of DNA dMTase by measuring both the release of CH_3 from the aqueous phase and the rate of generation of nonmethylated cytosine. As observed in Figs. 3A and 3B, the DNA dMTase maintains its initial velocity up to 90 minutes and the reaction continues up to 120 minutes. This time course is inconsistent with dependence on enzyme-bound additional nonreplenishable substrates such as dCTP or ATP or a nonreplenishable redox factor such as NADH or NADPH. Exhausting the nonreplenishable substrate or redox factor would have resulted in rapid deacceleration of the initial velocity. The results shown in Fig. 3 further exclude the remote possibility that a small number of dCTP molecules copurify with the enzyme and serve as substrate in a replacement reaction. The kinetic parameters of the enzyme do not support a model that the enzyme serves as a suicide substrate either since such a mechanism is inconsistent with a constant velocity. Defining the initial rate conditions enables the calculation of the Km of the enzyme for DNA as described in the experimental procedures and presented in Fig. 4 and Table 2.

Table 1

Purification of DNA dMTase

Purification step	Total protein (μg)	Specific activity (U/mg)	Fold purification
Nuclear extract	2064.0	0.2	-
Microcon 100 retentate	98.56	2.87	14.25
DEAE-Sepharose	60	365	1825
S-Sepharose	19	709	3285
Q-Sepharose	6.88	776	4245
DEAE-Sephacel	2.81	859	5360

A549 prepared nuclear extract (1 ml, 1.1 mg) was passed through a Microcon™ 100 spin column, the retainant was diluted to a conductivity equivalent to 0.2 M NaCl in buffer L and applied sequentially onto a DEAE-Sepharose™, S-Sepharose™, Q-Sepharose™ and DEAE-Sephacel™ column chromatography. The specific activity of the active fractions after each purification step is represented as U/ mg of protein. One unit of DNA dMTase equals the amount of protein which removes one picomole of CH_3 residue per hour from methylated mdCpdG substrates (described in the experimental procedures). The the ratio of specific activity of dMTase after each step to that of Microcon™ 100 retainant is presented as fold purification.

Table 2

Kinetic parameters for DNA dMTase

Method	K_m (DNA)	V_{max}
Cytosine formation	79.8 nM	32.26 pMole/h
CH_3 group elimination	72 nM	35.71 pMole/h
Hemi-methylated DNA	157.2 nM	34.52 pMole/h

The kinetic parameters Km and Vmax were calculated from the double reciprocal plots presented in Fig. 4 of mdCpG demethylase assays measuring either cytosine formation (using [mdC^{32}pdG]n as a substrate) or removal of CH_3 (using [^{3}H]-CH_3-[mdCpdG]n oligomer).

The initial velocity of DNA dMTase was determined at a substrate concentration range of 0.01 to 0.25 μM using either [mdC^{32}pdG]n double stranded oligomer (A) or [^{3}H]-CH_3-[mdCpdG]n as substrates (B) (Fig. 4).

The velocity versus substrate concentration plot approaches saturation at higher concentrations of DNA consistent with first order kinetics of the

reaction with respect to methylated DNA (Fig. 1F). The Km was determined by double reciprocal plots of velocity versus substrate concentration. A similar Km for DNA was calculated by either measuring the rate of generation of cytosine using the [mdC^{32}pdG]n substrate or by measuring the rate of removal of the methyl group using the ^{3}H labeled [mdCpdG] DNA. The Km for methylated DNA is in the micromolar range which is similar in range to the Km of DNA MeTase to hemimethylated DNA (Flynn et al., 1996; Glickman et al., 1997) suggesting a relatively high affinity of the enzyme to its substrate. These results further support the conclusion that DNA dMTase is an enzyme showing first order kinetics with respect to methylated DNA.

The purified DNA dMTase does not exhibit nuclease or glycosylase activity.

The experiments described in the experimental procedures demonstrate that DNA dMTase demethylates DNA by genuine removal of the methyl group and does not replace it with exogenous dCTP. To further exclude the possibility that our DNA dMTase removes methylation by a base or nucleotide replacement mechanism, we determined whether the purified DNA dMTase exhibited any nuclease or glycosylase activity. The purified DNA dMTase was incubated with a [^{32}pmdCpdG]n substrate which included a labeled ^{32}P 5' to mdC as described above. If DNA dMTase bears a glycosylase or nuclease activity α^{32}P-labeled residue(s) (deoxyribose-phosphate, the phosphate or the nucleotide) should be cleaved from the DNA by dMTase. The reaction mixture was loaded onto a TLC plate at different time points and separated in one dimension. As a control, the same substrate was incubated with A549 nuclear extract. As

observed in Fig. 5, the DNA remains intact following incubation with DNA dMTase while incubation of the same DNA with nuclear extracts results in release of dCMP and other labeled residues from the DNA.

Purified DNA dMTase (30 µl) was incubated with 1 nanogram of [^{32}pmdCpdG]n substrate in 50 µl total reaction volume containing buffer L and an aliquot of 3µl was withdrawn at different time intervals as indicated and analysed for the presence of excised ^{32}P residues by TLC. As a control, the DNA was incubated for 120 minutes with A549 nuclear extract. ^{32}P-α-dCTP was run as a control for the absence of nonincorporated dCTP in the labelled DNA as indicated. To demonstrate that DNA dMTase demethylated the DNA in this reaction the reaction products were digested with a 5' venom phosphodiesterase (+ V PDS) after 2 hours of incubation in the presence or absence of 100µg/ml RNase as indicated. [^{32}pmdCpdG]n that was incubated in the absence of DNA dMTase (ME) and nonmethylated [^{32}pdCpdG]n (NM) were run as controls.

Purified DNA dMTase was incubated with [mdC^{32}pdG]n substrate and aliquots were withdrawn at different time intervals and subjected to TLC analysis and venom phosphodiesterase treatment as described in the experimental procedures.

This is expected since nuclear extracts obviously contain a number of glycosylases and nucleases. Similarly, the DNA dMTase does not release any labeled residue from our standard methylated substrate where the labeled group is 3' to the m5dC (Fig. 5). The results in Fig. 5 also demonstrate that our method of preparation of labeled DNA effectively purify it from any nucleotide contamination. This suggests that the purified DNA dMTase does not bear any 3' or 5' nuclease activity. A glycosylase driven

mechanism of demethylation requires the presence of a nuclease to remove the apyrimidinic derivative. To demonstrate that demethylation activity was present in the absence of removal of a labeled residue from DNA, the 5' labeled mdC substrate was digested with a 5' mononuclease. As observed in Fig. 5, a significant percentage of mdCs are demethylated without any detectable release of a labeled residue from DNA. This experiment conclusively demonstrates that DNA dMTase does not bear a nuclease activity and that demethylation by DNA dMTase does not involve replacement of the mdC.

The product of the demethylation reaction is dCytosine

The results presented above show that DNA dMTase generates dC from mdC. This conclusion is based on the comigration of the product of DNA dMTase reaction with a standard 3' dCMP on TLC. The formal possibility exists however that the product of the reaction is a different derivative of dCMP that coincidentally comigrates with dCMP. To exclude this possibility, we subjected DNA dMTase treated DNA to remethylation with the CpG MeTase M. SssI (New England Biolabs). Only if the product of the DNA dMTase reaction is dC, it will serve as a substrate for the M. SssI MeTase. The DNA dMTase treated DNA was divided in two. The first aliquot was a control for demethylation by DNA dMTase. The second aliquot was reacted with M. SssI and then both aliquots were digested by a 3' mononuclease and analyzed by TLC. The results presented in Fig. 6 show that the demethylated product of DNA dMTase is dC since it is completely remethylated with M. SssI.

Purified DNA dMTase (30 µl) was incubated with 1 nanogram of [^{32}pmdCpdG]n substrate in 50 µl total reaction volume containing buffer L and divided into

two 20 µl aliquots. One aliquot was subjected to methylation with 10 units of SssI methylase (+dMTase +M. Sssl). The remethylated (+dMTase +M. Sssl) aliquot, nonremethylated aliquot (+dMTase), [^{32}PmdCPdG]n (ME) substrate that was not treated with DNA dMTase and a nonmethylated [^{32}pmdCpdG]n substrate (NM) were digested to 3' mononucleotides and analyzed by TLC.

Demethylation involves transfer of a hydrogen from water to regenerate cytosine

If demethylation involves removal of the methyl moiety from mdC, a hydrogen has to be transferred to the carbon at the 5' position to regenerate cytosine. Since no redox factors are involved, what is the source of the hydrogen? To test the hypothesis that the source of the hydrogen is water, we incubated a non labeled [mdCpdG]n substrate with DNA dMTase in the presence of tritiated water. Following the reaction, the DNA dMTase treated DNA and non DNA dMTase treated control were digested to 3' dNMP and separated on TLC with standards for each of the 5 possible dNMPs. The amount of label in each of the spots corresponding to the different dNMPs was determined by scintillation counting. As observed in Fig. 7, the cytosine product of the DNA dMTase reaction is labeled with tritiated hydrogen demonstrating that demethylation involves the removal of the CH_3 group, possibly as CH4 (or methanol), and its replacement with a hydrogen originating from water. No label is detected in any of the other dNMPs or in dCMP in the non-DNA dMTase treated sample. This experiment provides compelling evidence for a bona fide demethylation.

Nonlabelled [mdCpdG]n double stranded methylated substrate as well as a nonmethylated [dCpdG]n double stranded substrate were incubated with DNA dMTase in a 50 µl reaction volume in the presence

of (20 mCi, 0.1 mCi /mol) tritiated water for 1 hour at 37ºC. Methylated DNA was incubated with tritiated water in the absence of DNA dMTase as a control. Following the reaction, the DNA dMTase treated DNAs and non DNA dMTase treated control were digested to 3' dNMP and separated by TLC with standards for each of the 5 possible dNMPs. The amount of label in each of the spots corresponding to the different dNMPs was determined by scintillation counting.

[mdCpdG]n double stranded DNA was incubated with DNA dMTase in the presence of tritiated water as described in the experimental procedures. The DNA was digested to 3' mononucleotides, separated by TLC and exposed to a tritium sensitive phosphoimaging plate (FUJIX BAS-TR2040). The position of cold dNMP standards is indicated. dCMP is labeled. The other labeled spot is a partially digested product which migrates identically to a similar partial observed with ^{32}P labeled [dC^{32}pdG]n digested with micrococcal nuclease.

Dinucleotide sequence specificity of DNA dMTase

Methylation of CpG dinucleotides is the most characterized modification occurring in genomic DNA (Razin and Riggs, 1980; Gruenbaum et al., 1981), however other methylated dinucleotides were identified using bisulfite mapping (Clark, et al., 1995). Is demethylase specific to CpG methylation or will it result in demethylation of other methylated dinucleotides. The known DNA MeTase is specific for CpG sequences (Gruenbaum et al., 1982). We synthesized two other possible fully methylated cytosine bearing oligomers [dmC^{32}pdA]n,[mdC^{32}pdT]n as described in the methods. [mdC^{32}pdC]n was not synthesized since this synthesis required $\alpha^{32}P$ labeled mdCTP which was not yet available or a CpC methylase which was not

available either. Following incubation with the DNA dMTase, the reacted DNA and DNA incubated in the absence of DNA dMTase were subjected to micrococal nuclease digestion and TLC analysis. The results presented in Fig. 8B reveal that mdCpdA and mdCpdT are not demethylated by DNA dMTase suggesting that similar to the MeTase, the DNA dMTase exhibits dinucleotide specificity and does not recognize all methylated cytosines.

Demethylation of hemimethylated [mdC^{32}pdG]n. [dCpdG]n (HM), [mdC^{32}pdA]n (CpA) and [mdC^{32}pdT]n (CpT). One nanogram of the indicated substrates as well as a double methylated substrate [mdC^{32}pdG]n (CpG) were incubated in the presence (30µl) or absence of DNA dMTase (indicated as control for CpT and CpA and ME for CpG) and were subsequently subjected to 3' mononucleotides and TLC analysis. Hemimethylated DNA was incubated with DNA dMTase in the presence or absence of RNase (100µg/ml). Non methylated [dC^{32}pdG]n served as a control for the position of dCMP.

The substrate used in the experiments described in the experimental procedures is a homopolymer of the dinucleotide mdCpdG which is an unusual methylated DNA substrate. To test the hypothesis that DNA dMTase is a general DNA dMTase activity that demethylates dCpdGs flanked by a variety of sequences which are distributed at different frequencies, we determined whether it could demethylate different methylated cytosine sites in a plasmid DNA [pBluescript SK(+)]. We methylated the plasmid *in vitro* at all dCpdG sites with M.SssI. As the plasmid is methylated *in vivo* with the *E. coli* DCM MeTase at dCmdCdA/dTdGdG sites and with the DAM MeTase at dGmdAdTdC sites (adenine methylated), we utilized the substrate to determine the sequence specificity of DNA dMTase to different methylated

sequences and bases located on the same DNA molecule which is similar to the situation *in vivo*. This experiment excludes the possibility that the inability of DNA dMTase to demethylate the poly [mdCpdA/T]n substrates reflects peculiarities of this unusual DNA substrate rather than the sequence specificity of the enzyme. Demethylation following incubation with DNA dMTase was assayed using methylation sensitive restriction enzymes which is a different assay from the direct demethylation analysis used in the experiments described in the experimental procedures. Demonstrating demethylation of DNA using different approaches strengthens our confidence that our conclusions do not reflect an unexpected idiosyncrasy of one assay. As demonstrated in Fig. 8A, following incubation of methylated pBluescript SK(+) with DNA dMTase, all CCGG sites located in the plasmid are demethylated as demonstrated by the sensitivity of all sites to HpaII and all CGCG sites are demethylated based on the full cleavage of the DNA with HhaI.

Demethylation of methylated SK

Plasmid pBluescript SK was methylated with M. Sssl methylase. DNA dMTase treated (methylated SK+dMTase) and nontreated plasmid (methylated SK) as well as nonmethylated SK plasmid were digested with EcoRII, DpnI, HhaI, and HpaII.

DNA dMTase does not demethylate methylated adenines in the sequence GATC since the plasmid remained sensitive to DpnI (which cleaves methylated GATC sites). The sensitivity to DpnI demonstrates that the plasmid did not replicate or undergo extensive repair excluding this possibility as an explanation for demethylation. CpA and CpT dinucleotide sequences residing in CCA/dTdGdG site are not demethylated since the plasmid remains resistant to EcoRII. This

experiment verifies that DNA dMTase demethylates CpGs residing in different sequence contexts, discriminates between cytosine and adenine methylation and between cytosines located in different dinucleotide contexts. The fact that DNA dMTase treatment results in regeneration of a mC-resistant restriction enzyme recognition sequence is consistent with the conclusion that the product of the demethylation reaction is a cytosine monophosphate that bears a phosphodiester bond with its 3' and 5' neighbors. This is in accordance with the conclusion derived using other experimental approaches as described in Figures 6 and 7. In summary, our results are consistent with the hypothesis that DNA dMTase is a general mdCpdG demethylating enzyme.

DNA dMTase demethylates hemimethylated as well as fully methylated DNA

Most of genomic DNA is methylated on both strands (80% of the genome is methylated; Razin and Szyf, 1984). A demethylation activity that mimics the conditions *in vivo* should also demethylate both strands of DNA. The substrate used in our study to purify DNA dMTase is methylated on both strands as described in the methods. Therefore, the DNA dMTase purified in our study can demethylate DNA methylated on both strands. However, a study by Paroush et al. (Paroush et al., 1990) has shown that demethylation of the two strands of the a-actin promoter proceeds in two separate steps, implying that either the demethylase can recognize both double methylated and hemimethylated strands of DNA or that a demethylase that specifically recognizes hemimethylated substrates exists. An RNA dependent demethylation activity that demethylates hemimethylated DNA has been recently shown to exist in these cells. Jost has described a 5-methyl cytosine DNA Glycosylase activity that could replace methylated

cytosines with nonmethylated cytosines in hemimethylated DNA (Jost et al., 1995). One possibility is that demethylation of hemimethylated activity is accomplished by these other previously described activities. Alternatively, the bona fide DNA dMTase identified in our study might be able to perform both activities. To prepare a hemimethylated substrate, we used a nonmethylated dinucleotide oligomer substrate [dCpdG]n as a template. The substrate was incubated with DNA dMTase and demethylation was assayed as described in the experimental procedures. A result of such an analysis presented in Fig. 8B shows that DNA dMTase can demethylate hemimethylated as well as a substrate methylated on both strands. The Km of DNA dMTase for hemimethylated DNA was determined by measuring the initial velocity of the reaction at different concentrations of substrate. The calculated Km is 2.62 mM which is two fold higher than the Km for DNA methylated on both strands (Table 2). It is unclear yet whether this small difference in affinity to the substrate has any significance in a cellular context.

DISCUSSION

Methylation patterns are most probably formed and maintained by a balance of methylation and demethylation events (Szyf, 1994). Since demethylation of methylated cytosines is a very difficult chemical reaction, it has been originally proposed that demethylation is a passive process which comes about by replication without methylation (Razin and Riggs, 1980). This model provides a simple explanation for the demethylation observed during development and cellular differentiation. As a consequence of this appealing model, most of the attention in the last two decades in the field has focused on the DNA MeTase

whereas the true enzymatic activities involved in demethylation remained a mystery. However, a series of observations led to the conclusion that active demethylation which is independent of DNA replication occurs in differentiating cells (Szyf et al., 1985; Razin et al., 1986) in culture as well as *in vivo* (Kafri et al., 1993) during development. Transient transfection assays have further established that both sequence specific as well as general active demethylation occurs without replication (Paroush et al., 1990; Razin et al., 1986).

However, since the prevailing consensus has been that true demethylation is chemically unlikely, alternative mechanisms were sought to explain active demethylation which were based on excision of the methylated base by a glycosylase activity and replacement of the nucleotide with a nonmethylated dCMP (Jost et al., 1995; Weiss et al., 1996). The original model of replication without methylation was replaced with a repair without methylation model. Jost et al. has identified and recently purified an activity that can correct hemimethylated DNA as well as GT mismatches (Jost et al., 1995). The specificity of this activity to hemimethylated DNA might suggest that this enzyme is involved in repair of methylation errors as well as GT mismatches rather than altering and forming new patterns of methylation. More recently Weiss et al. (1996) have identified a novel RNA dependent activity that can replace a methylated dCpdG dinucleotide with a nonmethylated dCpdG dinucleotide that is probably covalently attached to the RNA molecule.

The present invention addresses the longstanding and seminal question of whether an enzyme that can truly remove methyl groups and reverse the action of DNA MeTase exists in mammalian cells. It is

clear that our understanding of how methylation patterns are formed and how they play their respective biological roles is essentially inadequate as long as the other partners of the machinery responsible for methylation are unknown. In this paper we demonstrate that a general mdCpdG bona fide demethylase exists and that this novel enzyme can perform the activities that are necessary for demethylation *in vivo*.

A number of previous observations formed the basis of our approach towards discovery of demethylase (DNA dMTase). The global demethylation observed in differentiating cells (Szyf et al., 1985) and *in vivo* (Brandeis et al., 1993) suggests that similar to DNA MeTase, the activity that demethylates DNA can in principle recognize any mdCpdG. This led to the use of a synthetic poly mdC^{32}pdG as a precisely defined substrate for DNA dMTase activity.

This substrate has some unique properties that are critical for the discovery of DNA dMTase. First, it is synthesized from a mdCTP precursor without a requirement for an enzymatic methylation step eliminating false positives that might have resulted from partial methylations. Second, the combination of a 5' labeled dGTP with a 3' mononuclease enables the very sensitive and direct detection of demethylation by thin layer chromatography simultaneously providing a very stringent assay for bona fide demethylation, since the radioactive detection of dCMP is only possible if it is ligated to the original ^{32}P labeled 3' dGMP. This ascertains that excision and replacement events and RNA dependent replacements of the mdCpdG will go undetected by this assay. Third, this substrate is absolutely methylated on both strands of DNA since the labeled strand is synthesized with mdCTP on a template that is 100% methylated. Whereas

methylation with most DNA MeTases should result in methylation of both strands, this is never certain and most methylation sensitive restriction enzymes can not differentiate between DNA that is methylated on one or both strands. Resorting to a strictly double stranded methylated DNA selects against activities that repair hemimethylated or mismatched DNA and favors activities that can alter an established pattern of methylation by true demethylation. The paradigm used for the synthesis of the $mdC^{32}pdG$ substrate was readily modified to synthesize other defined dinucleotide mdC substrates as well as a hemimethylated $dC^{32}pdG$ substrate. These well defined substrates enabled accurate comparisons of DNA dMTase activities.

Another previous observation that is critical is the observation that an important oncogenic pathway can induce DNA dMTase activity (Szyf et al., 1995; Szyf, 1994). Since global demethylation occurs only at discreet time points in development this limits the potential source material for DNA dMTase. The observation that cancer cells such as A549 bear relatively high levels of DNA dMTase activity pointed out an important cellular source for DNA dMTase.

Using A549 cells and the substrates described in the experimental procedures we were able to identify for the first time a novel enzyme that has not been discovered to date in any organism. This enzyme can remove a methyl group from a methyl-cytosine in DNA (Fig. 1, Fig. 2) and generate a fully functional nonmethylated cytosine (Fig. 6, Fig. 7 and 8). The demethylation of mdCMP does not take place unless they reside in DNA (unpublished data). Thus , this enzyme performs the reverse reaction to DNA methyltransferase and is an excellent candidate to be one of its important partners in shaping the methylation pattern

of genomes. It remains to be seen however whether lower organisms that bear DNA MeTases also harbor DNA dMTases or whether this activity is unique to organisms where DNA demethylation plays a critical role in development and regulation of genome function.

A number of approaches have been used to verify that the DNA dMTase activity identified is indeed a bona fide DNA dMTase and is not similar or homologous to any of the excision and replacement activities previously described to cause demethylation. The following lines of evidence suggest that DNA dMTase is not an excision-replacement activity . First, the activity was purified to almost homogeneity without loss of activity (Fig. 1A, B, C). The active purified fraction migrates as one distinct band excluding the possibility that a complex of proteins is involved (Fig. 1C). An excision replacement activity will require the participation of a glycosylase, AP nuclease, DNA polymerase and DNA ligase. Second, an excision replacement mechanism will require the presence of dCTP and ATP to proceed. DNA dMTase reaction does not require dCTP or ATP. Even if some dCTP or ATP might be present in the nuclear extracts, they should have been diluted out by the sequential chromatography steps (Fig. 1B). However, there is no increased requirement for exogenous supplementation of these factors through the purification steps. We have demonstrated that the first DEAE column purifies away any residual dCTP that might have been bound to proteins in the nuclear extract (Fig. 1B). On the other hand, the kinetic parameters of the DNA dMTase reaction exhibiting constant velocity for a long period of time exclude the possibility that dCTP which is bound to the active site of the enzyme is a source for dCTP (Fig. 3). Dependence of the reaction on bound

dCTP would have resulted in its rapid deacceleration after one round of demethylation. Third, the excision-replacement mechanism proposed by Jost et al. requires the activity of DNA polymerase b (Jost et al., 1995). The DNA dMTase reaction described here is not inhibited by very high concentrations of ddCTP (Fig. 2). Fourth, inclusion of high concentrations of mdCTP should compete with the residual dCTP for the replacement reaction and essentially inhibit demethylation but mdCTP does not inhibit DNA dMTase (Fig. 2). Fifth, the excision-replacement activity identified by Jost et al. is inhibited by RNase whereas the activity described here is augmented by RNase (Figs. 2A and 2C).

Three lines of evidence suggest that DNA dMTase is not the RNA dependent proteinase-insensitive activity identified by Weiss et al. (Weiss et al., 1996). First, DNA dMTase is activated and not inhibited by RNase (Figs. 2A, and 2C). Second, DNA dMTase is a protein and is absolutely inhibited by proteinase K treatment (Figs. 2A and 2C). Third, the product of the reaction is an intact nonmethylated cytosine (Fig. 1A, Fig. 6, Figs. 7 and 8) and not a mdCpdG dinucleotide which is the product of the reaction described by Weiss et al. (1996).

The strongest evidence that DNA dMTase is a bona fide demethylase is the fact that the DNA dMTase reaction releases the methyl group as a volatile residue. DNA dMTase releases tritiated methyl groups from [^{3}H]mdCpdG DNA into the volatile phase in a proteinase sensitive, time dependent and substrate concentration dependent manner (Fig. 2A and 2C, Fig. 3, Fig. 4). Released tritiated methyl residues could be detected in the gaseous phase (Fig. 1E). The kinetic parameters of the release of methyl residues are

similar to the values calculated for generation of cytosine (Table 2). Double labeling of the cytosine and the methyl group in mdC shows that the methyl group is released by the DNA dMTase whereas the hydrogen at the 6th position of cytosine remains intact (Fig. 1D). The other product of DNA dMTase reaction is nonmethylated cytosine because DNA dMTase treated DNA could be remethylated with M.SssI (Fig. 6) and digested with nonmethylated CpG recognizing enzymes (Fig. 8A). Finally, the DNA dMTase released methyl group at the 5 position is replaced with a labeled hydrogen originating from tritiated water to generate cytosine as predicted if a true demethylation reaction takes place (Fig. 7). Such labeling of cytosine with a hydrogen derived from tritiated water is not observed when the substrate is incubated in tritiated water in the absence of DNA dMTase or when a nonmethylated substrate is used. This suggests that labeling is enzymatic and specific to methylated DNA. As only the cytosine is methylated but not the mdC, it suggests that labeling occurs after mdC is demethylated. This labeling of cytosine by hydrogen could only occur if the methyl group per se is removed from the 5' position of cytosine leaving a free site in the carbon at the 5 position that generates a covalent bond with the water-derived hydrogen. This data provides forceful evidence that DNA dMTase removes the methyl group per se and generates cytosine with water derived hydrogen.

What is the chemical form by which the methyl group leaves the DNA? One possibility that has precedence in non-DNA demethylation is that the methyl group in mdC is oxidized and the leaves as methanol, CO_2 or formaldehyde. Our evidence suggests that the reaction is not enhanced by redox factors (Fig. 2B) and

that CO_2 or formaldehyde are not generated which is consistent with the lack of dependence on redox factors. One attractive possibility is that the methyl group leaves as methane. There is precedence for generation of methane from the methyl group residing at the 5' position of pyrimidines. Radioactive thymidine has been shown to release methane spontaneously (Merwitz, 1980) thus suggesting that removal of the methyl group per se is feasible if adequate energy is provided. What is the source of energy that drives this reaction is unknown and awaits the cloning and recombinant expression of the cDNA. To fully identify the leaving residue using gas chromatography, larger amounts of product are required. This will be accomplished only when the cDNA is cloned and recombinantly expressed to high levels. Whatever the precise chemical structure of the leaving residue is, this set of experiments demonstrates that methyl group is removed from the DNA as a volatile residue; possibly either as methane or methanol and that it is replaced with a hydrogen which stems from water. The DNA dMTase reaction does not require any additional factors and cofactors leading to the suggestion that the principal participants of this reaction are water, the DNA dMTase and the methylated substrate.

The DNA dMTase is a good candidate for the general DNA dMTase enzyme predicted to exist in previous studies (Szyf, 1994; Szyf et al., 1995; Szyf, 1995). First, DNA dMTase recognizes both DNA methylated on both strands as well as hemimethylated DNA (Fig. 1, Fig. 8B and Table 2), thus it could alter established DNA methylation patterns since most DNA is methylated on both strands. This is different from previously published demethylation activities which demethylate hemimethylated DNA specifically (Jost et

al., 1995). Second the enzyme exhibits mdCpdG dinucleotide sequence specificity which is the main methylated sequence in vertebrate genomes (Figs 8A and 8B) (Razin and Riggs, 1980).

Third, it recognizes mdCpdG in a variety of sequence contexts as implied by its ability to demethylate all the HpaII sites on an *in vitro* methylated pBluescript SK(+) plasmid (Fig. 8A) as expected from a general demethylase.

We propose based on this data that similar to the DNA MeTase which is a general dCpdG methylation activity, the DNA dMTase is a general mdCpdG demethylation activity. It has been previously proposed that the balance between these two activities affects the methylation pattern of the genome (Szyf, 1994). A general enzyme can be regulated by basic cellular regulatory signaling pathways. Similar to the general DNA MeTase, the abundance of DNA dMTase has been suggested to be regulated by Ras (Szyf et al., 1995) and most probably by other signaling pathways. Thus the global pattern of methylation of the genome might be controlled by central cellular signals that control the relative abundance of these two activities (Szyf, 1994). The lack of very distinct sequence specificity and the potential for general regulation is consistent with the global demethylation previously observed at specific points in cellular differentiation (Razin et al., 1984; Szyf et al., 1985; Razin et al., 1986), development (Brandeis et al., 1993) and oncogenesis (Feinberg and Vogelstein, 1983).

Methylation patterns exhibit site specificity, one remaining question is how can a general enzyme demethylate certain sites and not others? One possible hypothesis that has previously proposed is that an interplay between local factors such as transcription

factors (Szyf, et al., 1990; Szyf, 1991; Szyf, 1994) and the general availability of DNA dMTase and MeTase determines the pattern of methylation. The identification of DNA dMTase will enable future experiments to test this hypothesis.

The existence of a general DNA dMTase(s) adds unexpected potential plasticity to methylation patterns. It has been previously believed that changes in DNA methylation could only occur in development because loss of methylation could only occur during replication (Razin and Riggs, 1980). The presence of a general enzyme that could remove well established methylation patterns and the potential for modulation of its activity by general signals (Szyf et al., 1995) points out to the possibility that the covalent structure of the genome could be modified postmitotically in response to extracellular signals, pathological as well as physiological. Whereas future experiments will determine whether the DNA dMTase is regulated and whether it could be activated in post mitotic as well as mitotic cells, the experiments described in this manuscript lay out the methodology to assay this activity and test this hypothesis.

One of the confusing issues in the understanding of the role of methylation in cancer development is the fact that both global hypomethylation (Feinberg and Vogelstein, 1983) as well as hyperactivation of DNA MeTase (El-Deiry et al., 1991) are described in cancer cells. One possible way to resolve this contradiction is to propose that both DNA MeTase and DNA dMTase are induced in cancer cells (Szyf, 1994; Szyf et al., 1995). One critical question to be addressed in the future is whether increased DNA dMTase is a component of oncogenic programs as has been previously hypothesized (Szyf, 1994: Szyf et al.,

1995). In this context it is important that bona fide DNA dMTase activity was found in cancer cells.

What is the relationship between this DNA dMTase activity and the two other demethylation activities previously described. It is possible that preventing aberrant methylation events is critical for survival and therefore alternative strategies have been developed to avoid this situation. Different DNA dMTases operating by different mechanisms might function in guarding the methylation pattern from change versus those that alter established methylation patterns. It is possible that demethylation activities that target specifically hemimethylated DNA are primarily focused on correcting methylation errors. Alternatively, the different demethylation activities might act at different stages of cellular development. The DNA dMTase described here is found in cancer cells whereas previously described activities were described in differentiating cells (Weiss et al., 1996; Jost et al., 1995; Fremont et al., 1997). One other interesting issue is whether DNA dMTase is unique to vertebrates or whether other lower organisms bear DNA dMTase activities which might have different and unexpected biological roles.

It is clear that many future experiments are required to resolve these issues. However, the finding of a bona fide DNA dMTase described in this paper opens new directions in the continuing effort to unveil the perplexing biological role of DNA methylation.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and

including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

REFERENCES

1. Bechtold, M.M. et al. (1972) J. Biol. Chem. 247, 7650-7656
2. Benvensity, N. et al. (1985) Proc. Natl. Acad. Sci. USA 82, 267-271
3. Brandeis, M. et al. (1993) Bioassays 15, 709-713
4. Clark, S.J. et al. (1995) Nature Genet. 10, 20-27
5. Dong, Q. et al. (1993) J. Biol. Chem. 268, 24163-24174
6. El-Deiry, W.S. et al. (1991) Proc. Natl. Acad. Sci. USA 88, 3470-3474
7. Feinberg, A.P. et al. (1983) Nature 301, 89-92
8. Fisher, P. A. et al. (1979) J. Biol. Chem. 254, 6128-6137
9. Flynn, J. et al. (1996) Biochemistry35, 7308-7315
10. Frank, D. et al. (1991) Nature 351, 239-241
11. Glickman, J.F. et al. (1997) Biochem. Biophys. Res. Comm. 230, 280-284
12. Gruenbaum, Y. et al. (1981) FEBS Lett. 124, 67-71
13. Gruenbaum, Y. et al. (1982) Nature 295, 620-622
14. Holliday, R. et al. (1991) Somat. Cell. Mol. Genet. 17, 537-542
15. Huszti, S. et al. (1986) FEBS Lett. 209, 362-366
16. Jost, J. P. et al. (1995) J. Biol. Chem. 270, 9734-9739
17. Kautiainen, T.L. et al. (1986) J. Biol. Chem. 261, 1594-1598
18. Merwitz, O. (1980) Int. J. Radiat. Biol. 38, 53-61
19. Monk, M. (1995) Dev. Genet. 17, 188-197
20. Nan, X. et al. (1997) Cell. 88, 471-481
21. Paroush, Z. et al. (1990) Cell 63, 1229-1237
22. Razin, A. et al. (1980) Science 210, 604-610
23. Razin, A. et al. (1984) Biochim. Biophys. Acta. 782, 331-342
24. Razin, A. et al. (1984) Proc. Natl. Acad. Sci. USA 81, 2275-2279
25. Razin, A. et al. (1986) Proc. Natl. Acad. Sci. USA 83, 2827-2831
26. Razin, A. et al. (1995) Human Mol. Genet.,4, 1751-1755

27. Razin, A. and Cedar, H. (1991) Micorbiol. Rev.55, 451-458

28. Rouleau, J. et al. (1992) J. Biol. Chem. 267, 7368-7377

29. Rouleau, J. et al. (1995) J. Biol. Chem. 270, 1595-1601

30. Rudnicki, M.A. et al. (1987) in Teratocarcinomas and embryonic stem cells a practical approach. (Robertson, E.J., eds.) pp. 19-49, IRL Press, Oxford, Washington

31. Saluz, H.P. et al. (1986) Proc. Natl. Acad. Sci. USA 83, 7167-7171

32. Sauer, F.D. et al. (1977) Biochem. Biophys. Res. Comm. 79, 124-132

33. Shemer, R. et al. (1991) J.Biol. Chem. 266, 23676-23681

34. Stein, R. et al. (1982) Proc. Natl. Acad. Sci. USA 79, 61-65

35. Szyf, M. et al. (1984) Proc. Natl. Acad. Sci. USA 81, 3278-3282

36. Szyf, M. et al. (1985) Proc. Natl. Acad. Sci. USA 82, 8090-8094

37. Szyf, M. et al. (1990) Mol. Cell. Biol. 10, 4396-4340

38. Szyf, M. (1991) Biochem. Cell Biol. 69, 764-767

39. Szyf, M. et al. (1991) J. Biol. Chem. 266, 10027-10030

40. Szyf, M.(1994) Trends Pharmacol. Sci. 7, 233-238

41. Szyf, M. et al. (1995) J. Biol. Chem. 270, 12690-12696

42. Szyf, M. (1996) Pharmacol. Ther. 70, 1-37

43. Toews, M. et al. (1979) J. Biol. Chem. 254, 1761-1764

44. Yisraeli, J. and Szyf, M. (1984) in DNA Methylation and its Biological Significance. Springer Verlag Inc. N.Y. (Razin, A et al. eds.) pp 353-378

45. Vairapandi, M. et al. (1993) *Nucl. Acids Res.* 21, 5323-5327

46. Weibauer, K. et al. (1990) Proc. Natl. Acad.Sci. USA 87, 5842-5845

47. Weiss, A. et al. (1996) Cell 87, 709-718

WHAT IS CLAIMED IS:

1. A DNA demethylase enzyme having about 200 to about 240 Kbp, and wherein said DNA demethylase enzyme is overexpressed in cancer cells.

2. Use of an antagonist or inhibitor of DNA demethylase for the manufacture of a medicament for cancer treatment, for restoring an aberrant methylation pattern in a patient DNA, or for changing a methylation pattern in a patient DNA.

3. Use according to claim 2, wherein said antagonist is a double stranded oligonucleotide that inhibits demethylase at a Ki of 50nM.

4. Use according to claim 3, wherein said oligonucleotide is $\begin{bmatrix} C^mGC^mGC^mGC^mG \\ G^mCG^mCG^mCG^mC \end{bmatrix}_n$.

5. Use according to claim 2, wherein the inhibitor comprises an anti-DNA demethylase antibody or an antisense of DNA demethylase.

6. Use according to one of claims 2 or 5, wherein the change of the methylation pattern activates a silent gene.

7. Use according to claim 6, wherein the activation of a silent gene permits the correction of genetic defect.

8. Use according to claim 7, wherein said genetic defect is β-thalassemia or sickle cell anemia.

9. Use of the demethylase of claim 1, for removing methyl groups on DNA in vitro.

10. Use of the demethylase of claim 1 or its cDNA, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

11. Use of the demethylase of claim 1 or its cDNA, for inhibiting methylation in cancer cells using vector mediated gene therapy.

12. An assay for the diagnostic of cancer in a patient, which comprises determining the level of expression of DNA demethylase of claim 1 in a sample from said patient, wherein overexpression of said DNA demethylase is indicative of cancer cells.

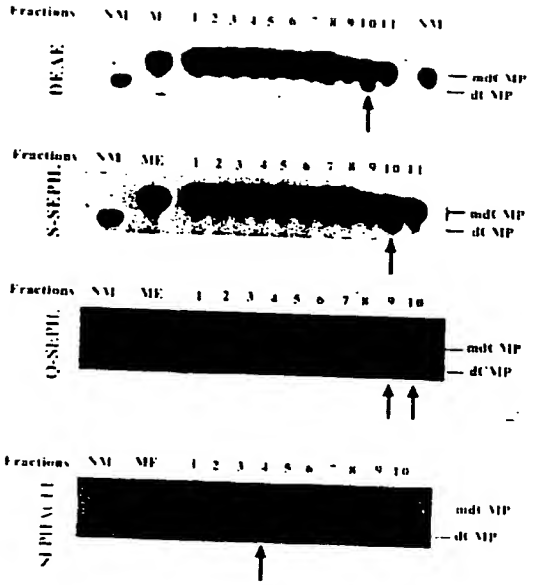
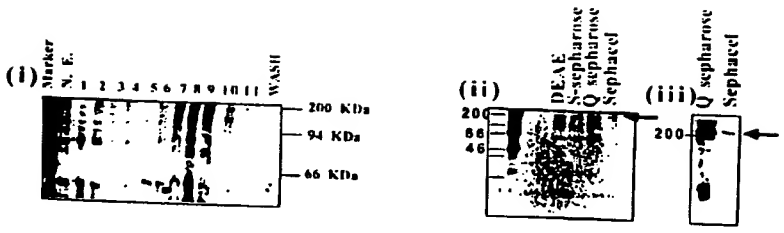

Fractions
DEAE
S-SEPH.
Q-SEPH.
SEPHACEL

Fig. 1A


(i)
Marker
N. E.
WASH
200 KDa
94 KDa
66 KDa
(ii)
DEAE
S-sepharose
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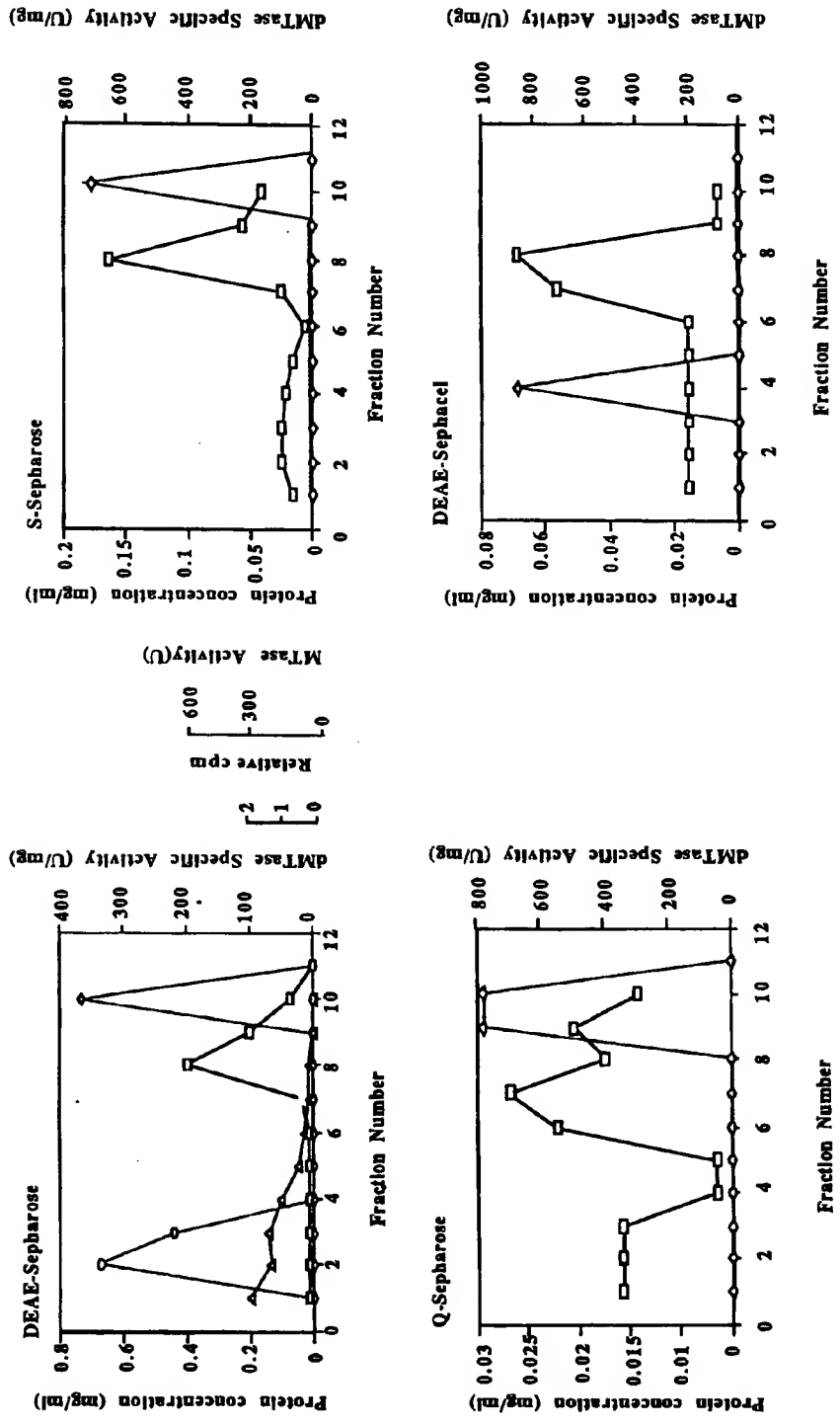
Sephacel
200
66
46
(iii)
Q sepharose
Sephacel
200

Fig. 1C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DEAE-Sepharose
DEAE-Sepharcel
Q-Sepharose
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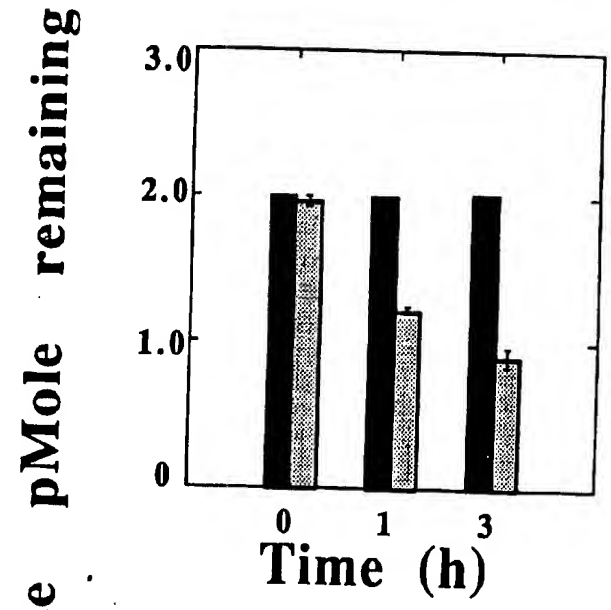
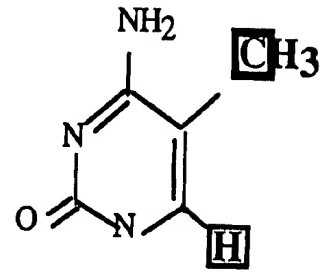
dMTase Specific Activity (U/mg)
Fraction Number
MTase Activity(U)
Relative cpm

Fig. 1B

D





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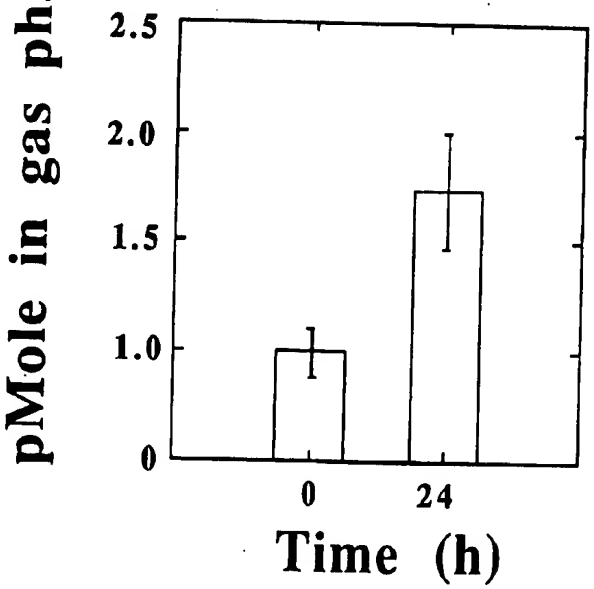

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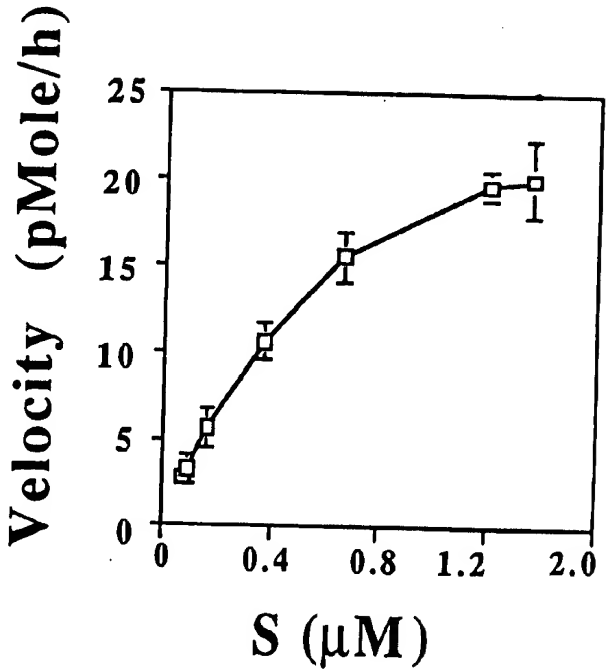
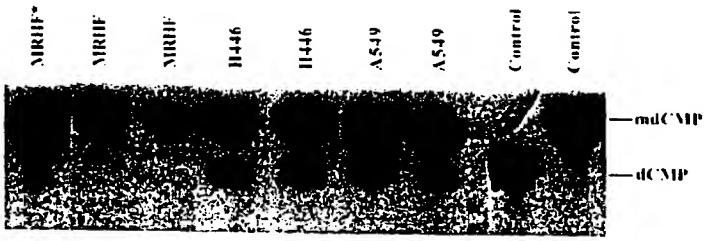


Fig. 1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MRHF
MRHF
H446
H446
A549
A549
Control
Control
mdCMP
dCMP

Fig. 1G

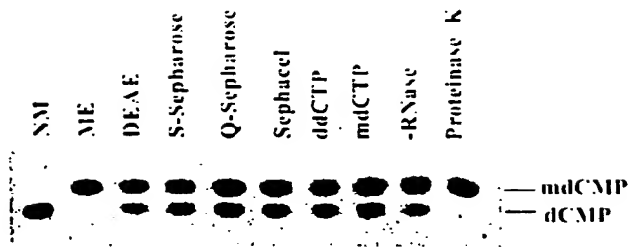
NM
ME
DEAE
S-Sepharose
Q-Sepharose
Sephacel
ddCTP
mdCTP
-RNase
Proteinase K
mdCMP
dCMP

Fig. 2A

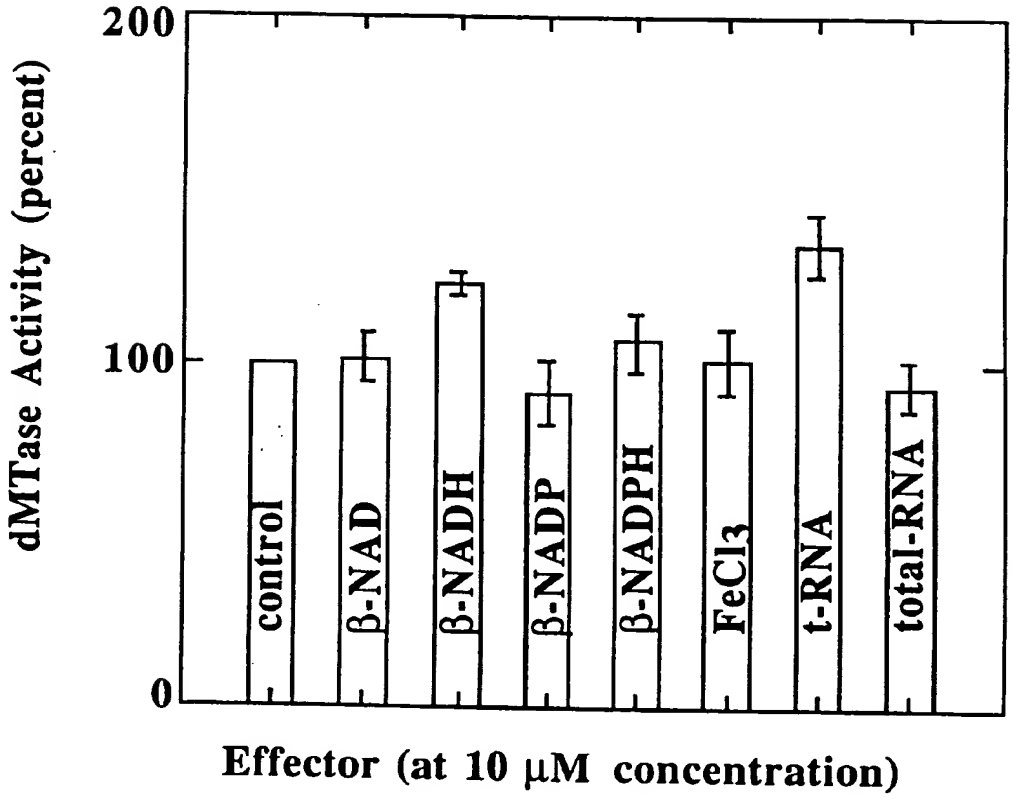
200
100
0
dMTase Activity (percent)
control
β-NAD
β-NADH
β-NADP
β-NADPH
FeCl3
t-RNA
total-RNA
Effector (at 10 μM concentration)

Fig. 2B

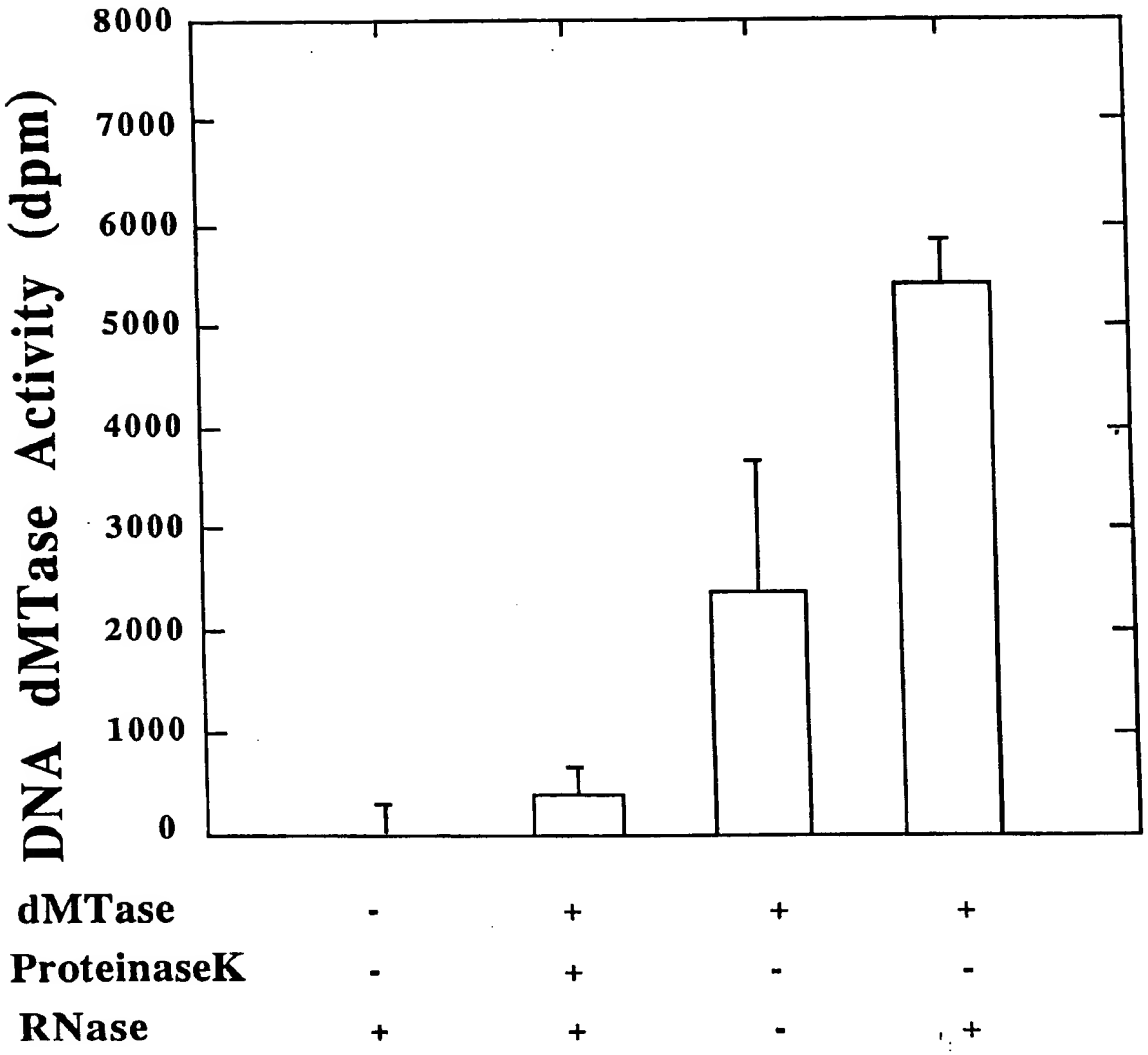
DNA dMTase Activity (dpm)
8000
7000
6000
5000
4000
3000
2000
1000
0
dMTase - + + +
ProteinaseK - + - -
RNase + + - +

Fig. 2C

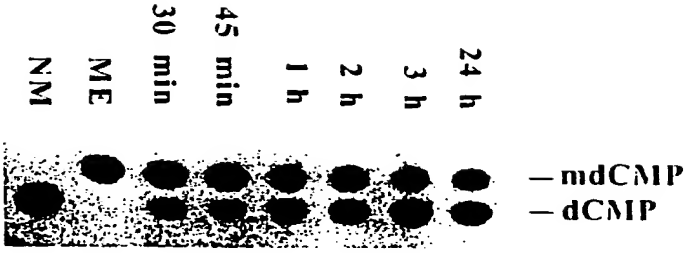
NM
ME
30 min
45 min
1 h
2 h
3 h
24 h
— mdCMP
— dCMP

Fig. 3A

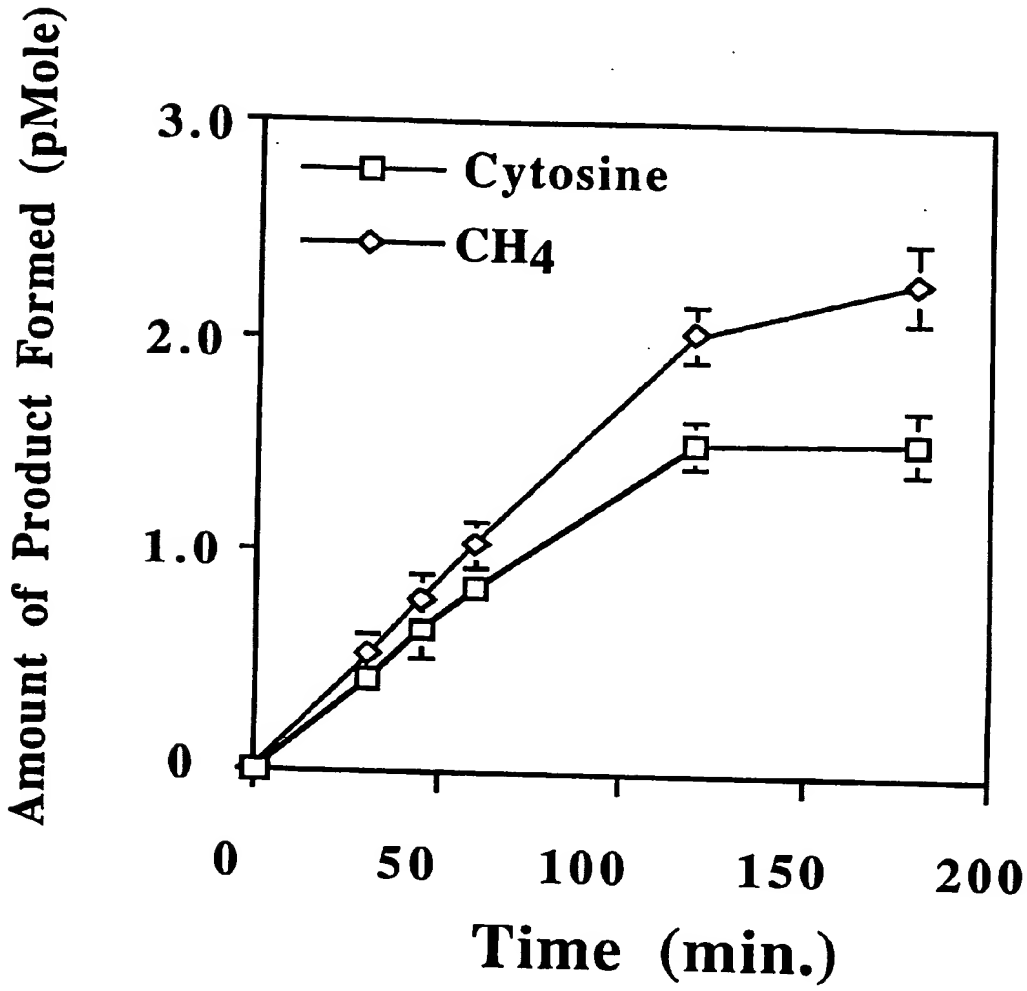
Amount of Product Formed (pMole)
3.0
2.0
1.0
0
Cytosine
CH4
0
50
100
150
200
Time (min.)

Fig. 3B

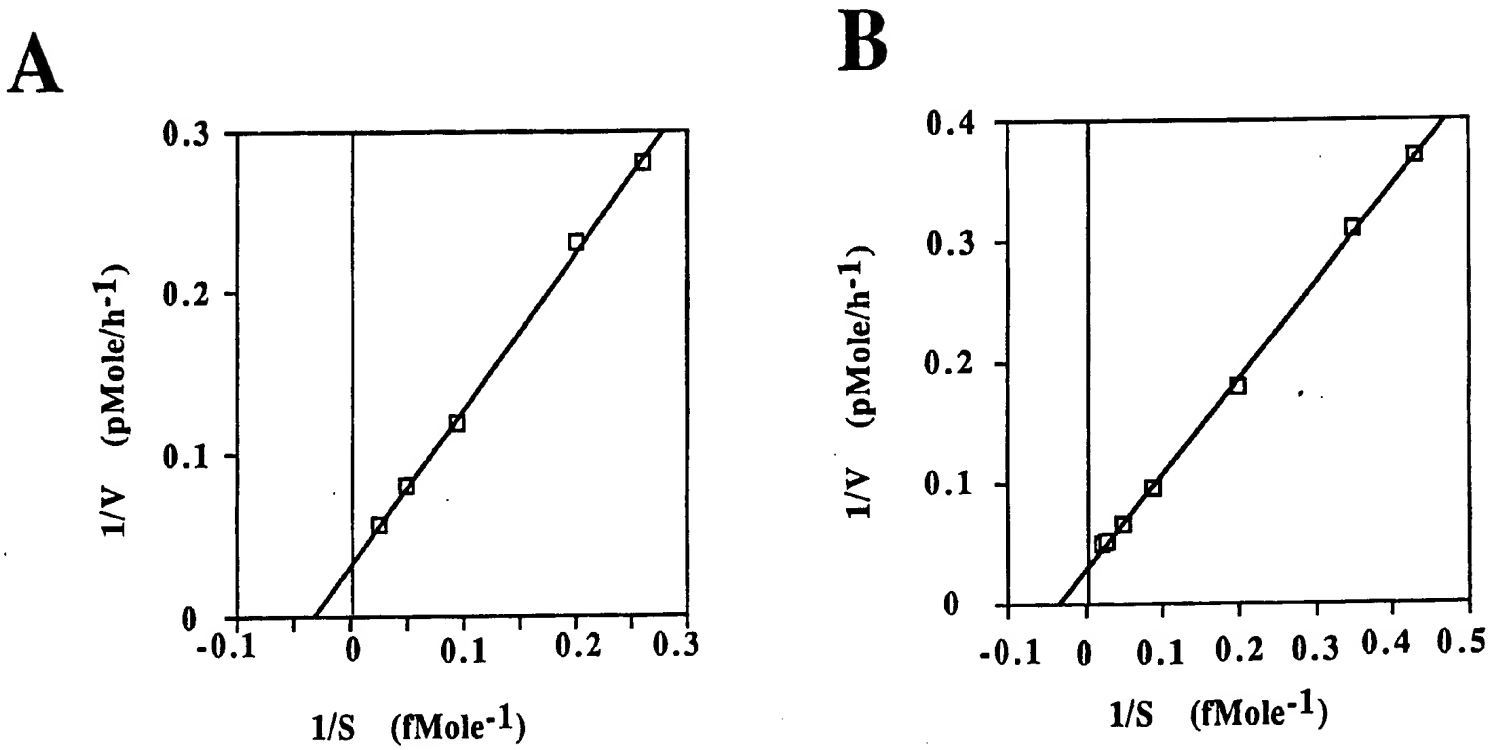

Fig. 4

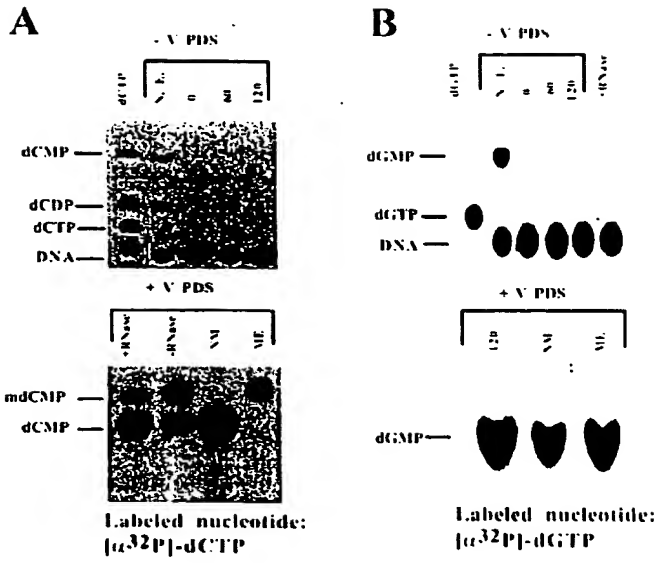
A
- V PDS
dCMP
dCDP
dCTP
DNA
+ V PDS
mdCMP
dCMP
Labeled nucleotide:
[α32P]-dCTP
B
- V PDS
dGMP
dGTP
DNA
+ V PDS
dGMP
Labeled nucleotide:
[α32P]-dGTP

Fig. 5

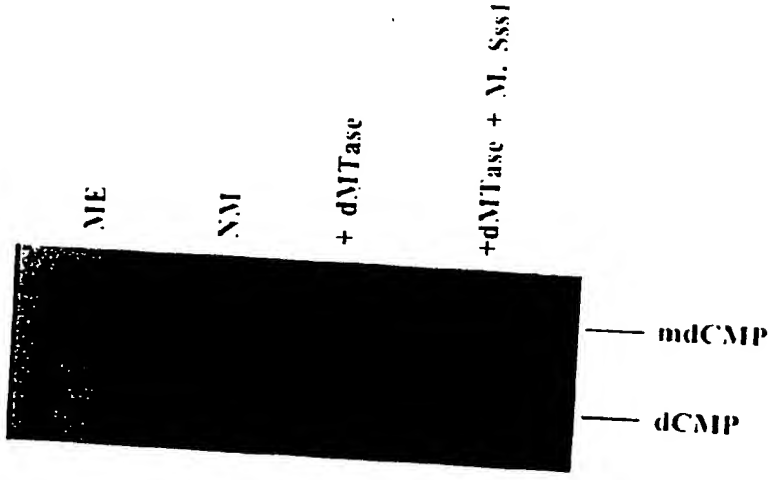
ME
NM
+ dMTase
+dMTase + M. SssI
mdCMP
dCMP

Fig. 6

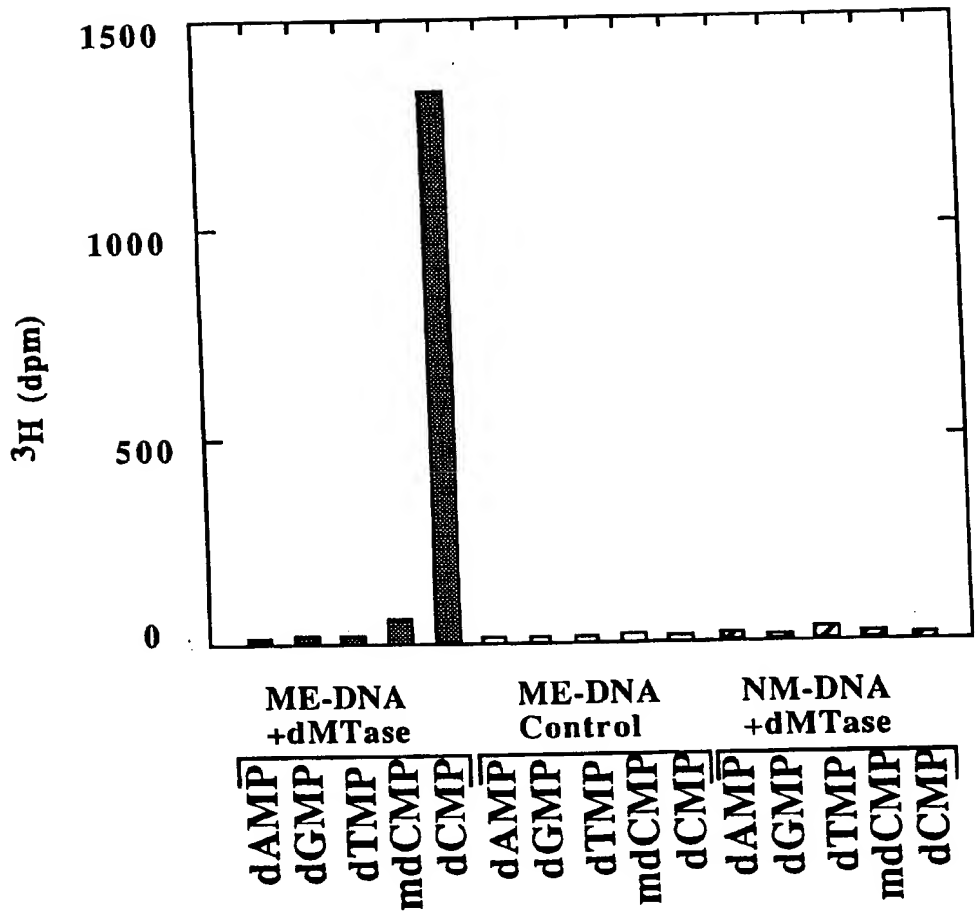
1500
1000
500
0
^{3}H (dpm)
ME-DNA +dMTase
ME-DNA Control
NM-DNA +dMTase
dAMP
dGMP
dTMP
mdCMP
dCMP
dAMP
dGMP
dTMP
mdCMP
dCMP
dAMP
dGMP
dTMP
mdCMP
dCMP

Fig. 7A

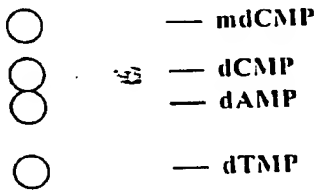
mdCMP
dCMP
dAMP
dTMP

Fig. 7B

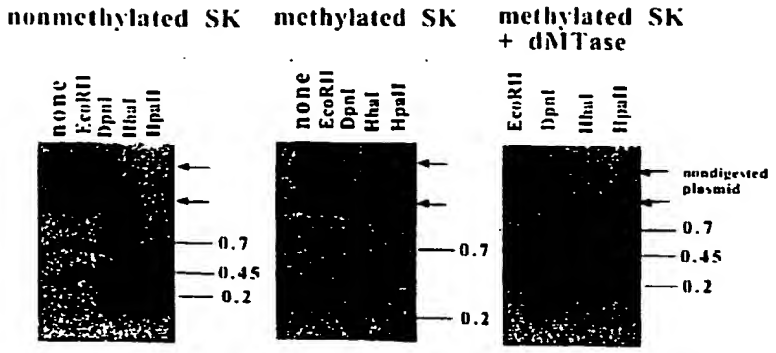
nonmethylated SK
methylated SK
methylated SK
+ dMTase
none
EcoRII
DpnI
HhaI
HpaII
0.7
0.45
0.2
nondigested
plasmid

Fig. 8A

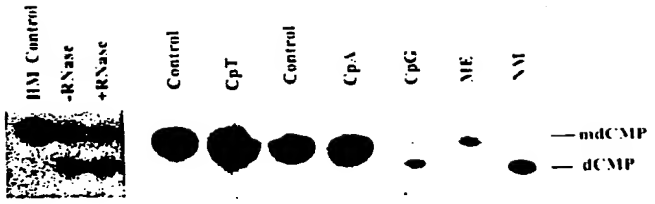
HM Control
-RNase
+RNase
Control
CpT
Control
CpA
CpG
ME
NI
mdCMP
dCMP

Fig. 8B